

# **Protecting the Safety and Quality of Live Oysters through the Integration of Predictive Microbiology in Cold Supply Chains**

by

Judith Fernandez-Piquer

MSc Food Safety, Wageningen University, 2007

BSc Food Science and Technology, University of Barcelona, 2006

BSc Technical Industrial Engineering, Polytechnic University of Catalonia, 2003

A thesis submitted to the School of Agricultural Science, University of Tasmania in fulfilment of the requirements for the degree of Doctor of Philosophy November, 2011



## **Declaration of originality and authority of access**

### **Declaration of Originality**

I, Judith Fernandez-Piquer, certify that this thesis does not contain any material which has been accepted for a degree or diploma by the University of Tasmania or any other institution, except by way of background information and duly acknowledged in the thesis. To the best of my knowledge and belief, this thesis does not contain material previously published or written by another person except where due reference is made in the text of the thesis and nor does this thesis contain any material that infringes copyright.

---

Judith Fernandez-Piquer, 30 November 2011

### **Authority of access**

This thesis may be made available for loan and limited copying in accordance with the Copyright Act 1968.

---

Judith Fernandez-Piquer, 30 November 2011

## Acknowledgements

This research has been possible with the collaboration of amazing people I have met along the way. After three and a half years of oyster adventures, I am glad to have the opportunity to express my gratitude to all of you.

I would like to offer my sincere gratitude to my supervisor, Mark Tamplin, for all the experience and knowledge you have shared with me. I thank your guidance, advice and your intensive review of different documents during my PhD including this thesis. I really appreciate having had the opportunity of discovering Tasmania while working with you.

I am also very grateful for the review of part of this thesis by my co-supervisors Tom Ross and John Bowman. I acknowledge Tom Ross for the insightful discussions, and also for your patience developing and optimizing the predictive program. I also thank John Bowman for your kind guidance during the molecular studies. Thanks to Tom McMeekin and June Olley for your friendly comments and wise suggestions during all this period.

Many thanks to all my colleagues at University of Tasmania, Ann Wanasen and Lyndal Mellefont for the professional and personal great moments shared on the island. A special thanks to Corina Ly and Steve Quarrell for your invaluable emotional support.

I am grateful to Tom Madigan, Damian May and Cath McLeod who collaborated on Pacific oyster studies. I thank Adam Smolenski for conducting fragment analyses. I also wish to thank Shane Powell for your advice during the genetic studies, David Ratkowsky for the fantastic statistics discussions and Silvia Estrada-Flores for your assistance in

preparing the stochastic model. Thanks to Bianca Porteus, Alison Dann and Chris Chapman “Chapito” for helping with oyster washing and drilling.

My gratitude to the Australian Shellfish Quality Assurance Program managers and the Department of Primary Industries in Tasmania, New South Wales and South Australia for support and participation in the project. I thank Wayne O'Connor, Michael Dove and Tony Troup for their help with the work undertaken with the New South Wales oysters; Ken Lee for organizing the oyster shipments from South Australia; Alison Turnbull and Ray Brown for providing information about the Tasmanian oyster industry; and Anthony Zammit and Rachel King for their enthusiastic interest in my research.

I acknowledge Christian Garland and Scott Parkinson who introduced me to oyster science and oyster industry. I would also like to thank Peter Kosmeyer and the rest of the Marine Culture Ltd oyster farm team in Tasmania for answering my many curiosities about oysters, for teaching me how to shuck oysters and kindly providing all samples at no cost.

I am deeply grateful to the Australian Seafood Cooperative Research Centre and the Australian Oyster Consortium for supporting financially this research, some of my training and my PhD scholarship. I also want to thank to University of Tasmania for my Endeavour International Postgraduate Research scholarship.

I am forever grateful for the love and encouragement of my family: Montse and Ramón :“*Gracias por entender mis ganas de experimentar nuevos destinos y estar a mi lado a pesar de la distancia*”, and Héctor: “*Gracias por entender mi ausencia*”.

Judith Fernandez-Piquer, Hobart, 2011

## Abstract

*Vibrio parahaemolyticus* is a bacterial species indigenous to marine environments and can accumulate in oysters. Some *V. parahaemolyticus* strains are pathogenic and seafood-borne outbreaks are observed worldwide. This pathogen can reach infectious levels in oysters if post-harvest temperatures are not properly controlled. The aim of this thesis was to support oyster supply chain management by developing predictive microbiological tools to improve the safety and quality of oysters in the market.

A predictive model was produced by injecting Pacific oysters (*Crassostrea gigas*) harvested in Tasmania with a cocktail of pathogenic and non-pathogenic *V. parahaemolyticus* strains, and measuring population changes over time at static storage temperatures from 4 to 30°C. In parallel, the total viable bacteria count (TVC) was measured.

The *V. parahaemolyticus* and TVC growth models were then evaluated with Pacific and Sydney Rock oysters (*Saccostrea glomerata*) harvested in New South Wales containing natural populations of *V. parahaemolyticus*. Oysters were stored at static temperatures from 15 to 28°C, and *Vibrio parahaemolyticus* and TVC viability were measured. In Pacific oysters, TVC growth was observed at all tested temperatures while *V. parahaemolyticus* growth was observed only at 23 and 28°C. In Sydney Rock oysters, TVC growth was observed only at 24°C and *V. parahaemolyticus* did not grow at any storage temperature tested. These interesting findings potentially indicate that Sydney

Rock oysters have enhanced anti-bacterial defences compared to Pacific oysters, and that commercial temperature controls to manage *V. parahaemolyticus* growth can be different.

Consistently higher growth rates of *V. parahaemolyticus* and TVC were observed in Tasmanian versus New South Wales oysters and may have been caused by different factors. They include variations in levels of background competitive flora, different growth rates among *V. parahaemolyticus* strains, and/or changes in the natural bacterial community structure influenced by conditions at the harvest site or during shipment to the laboratory. Nevertheless, the overall performance of the model was “fail-safe” for predicting growth of *V. parahaemolyticus* in Pacific oysters and would be a preferred public health tool.

The *V. parahaemolyticus* and TVC predictive models for Pacific oysters were integrated in an Excel<sup>®</sup> software tool. The model allows users to input time-temperature profiles and analyse the effects of dynamic storage temperatures normally found in oyster supply chains on bacterial growth. The tool was evaluated in five different simulated oyster supply chains (refrigerated and non-refrigerated). Observed and predicted *V. parahaemolyticus* and TVC growth rates were compared and a model over-estimation mean of 2.30 for *V. parahaemolyticus* and 2.40 for TVC were observed as determined by the bias factor index. Reasons for over-estimations are likely the same as those for model validation experiments.

Uncertainty and variability are associated with oyster supply chains. Therefore, a stochastic model which encompassed the operations from oyster farm to the consumer was built using ModelRisk<sup>®</sup> risk analysis software. This case study generated probabilistic distributions and the percentage of oysters containing *V. parahaemolyticus* and TVC

during each operation of the supply chain. The results were used for an objective evaluation of the influence of short and long supply chains during summer and winter seasons. The stochastic model can help the oyster industry evaluate the performance of oyster cold chains, and potentially enable real-time decisions if coupled with suitable traceability systems. It can also provide risk managers with valuable information about *V. parahaemolyticus* exposure levels.

Finally, in order to better understand microbial changes in oysters during distribution and storage, the dynamics of microbial communities in Pacific oysters was determined using 16S rRNA-based terminal restriction length polymorphism and clone library analyses. Significant differences in bacterial community composition were observed, and the predominant bacteria were identified for fresh and stored oysters at different temperatures. High microbial diversity in oysters was observed, with up to 73 different genera-related identified clones among all samples. The results identified *Psychrilyobacter* spp. as a potential spoilage indicator for future shelf-life studies, and *Polynucleobacter* and a bacterial group related to *Alkaliflexus* as possible indicators for storage temperature control in Pacific oysters. In future studies, quantitative correlations of the identified species and the freshness of oysters should be explored to determine whether the predominant microbes identified represent significant “specific spoilage organisms”, and to determine if they are antagonistic to human bacterial pathogens that are found in oysters.



## Statement of Co-Authorship

**Fernandez-Piquer J.**, Ross T., Bowman, J.P., Tamplin M.L., ‘Predictive Models for the Effect of Storage Temperature on Viability of *Vibrio parahaemolyticus* and Total Viable Bacteria Count in the Pacific Oyster (*Crassostrea gigas*)’. Applied and Environmental Microbiology, published online ahead of print on 14 October 2011 (AEM 05568-11).

**Fernandez-Piquer J.**, Ross T., Bowman, J.P., Tamplin M.L., ‘Influence of Storage Temperature on Microbial Communities in Live Pacific Oysters (*Crassostrea gigas*)’. Journal article in preparation.

**Fernandez-Piquer J.**, Ross T., Bowman, J.P., Estrada-Flores S., Tamplin M.L., ‘Stochastic Model for *Vibrio parahaemolyticus* and Total Viable Bacteria Count Management in the Pacific Oyster (*Crassostrea gigas*) Supply Chain’. Journal article in preparation.

The following specifies contributions of all authors and supervisors to the above listed manuscripts:

Mark Tamplin developed the concept and design of this thesis.

Mark Tamplin, John Bowman and Tom Ross assisted with the general supervision of this thesis. This included general advice, help with the interpretation of the data, and proof reading and contributing to the above listed manuscripts.

Judith Fernandez-Piquer conducted all experiments and measurement in this thesis. In Chapter 2, some of the kinetics measurements were performed by Tom Madigan, Damian May and Cath McLeod at SARDI. In Chapter 5, the fragment analyses were conducted by Adam Smolenski at the Central Science Laboratory.

In Chapter 3, Tom Ross developed the predictive program. In Chapter 4, Silvia Estrada-Flores assisted in preparing the stochastic model. In Chapter 5, advice during the study was provided by Shane Powell.

We the undersigned agree with the above stated “proportion of work undertaken” for each of the above submitted peer-reviewed manuscripts contributing to this thesis.

Supervisor:

Mark Tamplin

Candidate:

Judith Fernandez-Piquer

Co-supervisor:

John Bowman

Co-supervisor:

Tom Ross

## Presentations from this thesis

**Fernandez-Piquer J.**, Ross T., Bowman, J.P., Silvia Estrada-Flores, Tamplin M.L., ‘Stochastic model for safety and quality management in the Pacific oyster supply chain’, Australian Institute of Food Science and Technology, Securing global food safety, 26-28 September 2011, Melbourne, Australia [Poster and oral presentation, awarded prize]

**Fernandez-Piquer J.**, Ross T., Bowman, J.P., Tamplin M.L., ‘Influence of storage temperature on bacterial communities in live Pacific oysters (*Crassostrea gigas*)’, Australian Society for Microbiology, annual scientific meeting, 4-8 July 2011, Hobart, Australia [Oral presentation, BD student award]

**Fernandez-Piquer J.**, Ross T., Bowman, J.P., Tamplin M.L., ‘The importance of *Vibrio* bacteria on shellfish’, Actual Overview of Shellfish Sanitary Control, 6-8 April 2011, Ensenada, Mexico [Invited oral presentation and round-table discussion]

**Fernandez-Piquer J.**, Ross T., Bowman, J.P., Tamplin M.L., ‘Validation of a *Vibrio parahaemolyticus* predictive model in two different species of Australian oysters’, Vibrios in the Environment International Conference , 7-12 November 2010, Biloxi, USA [Poster presentation, Seafood CRC travel award]

**Fernandez-Piquer J.**, Ross T., Bowman, J.P., Tamplin M.L., ‘A tool to manage *Vibrio parahaemolyticus* in oysters’, Australian Shellfish Quality Assurance Program annual meeting , 26-28 October 2010, Port Lincoln, South Australia [Oral presentation]

**Fernandez-Piquer J.**, Ross T., Bowman, J.P., Tamplin M.L., 'Modelling *Vibrio parahaemolyticus* growth in Australian oysters', University of Tasmania's Postgraduate Research Conference 'Sharing Excellence in Research' , 2-3 September 2009, Sandy Bay Campus, Hobart, Tasmania [Oral presentation, awarded prize]

**Fernandez-Piquer J.**, Ross T., Bowman, J.P., Tamplin M.L., 'Viability of *Vibrio parahaemolyticus* in Australian Pacific Oysters', 7th International Conference Molluscan Shellfish Society , 14-19 June 2009, Nantes, France [Oral presentation]

*“All models are wrong but some are useful” George Box 1979*

*In the next pages, some useful but not perfect predictive microbiology models  
for the oyster industry are presented*



# Contents

Declaration of originality and authority of access .....	3
Acknowledgements .....	4
Abstract .....	6
Statement of Co-Authorship .....	9
Presentations from this thesis .....	11
Contents .....	14
List of abbreviations .....	17
List of tables .....	19
List of figures .....	21
1 – Introduction .....	24
1.1 Oyster industry in Australia .....	24
1.2 Microbiological safety of oysters .....	31
1.2.1 The genus <i>Vibrio</i> .....	32
1.2.2 <i>Vibrio parahaemolyticus</i> .....	34
1.3 Microbiological quality of oysters .....	43
1.3.1 Oyster spoilage .....	43
1.4 Supply chain management .....	47
1.4.1 Traceability .....	50
1.5 Predictive microbiology .....	54
1.5.1 Risk assessment .....	60
1.6 Objectives of this research .....	64

2 - Predictive models for the effect of storage temperature on viability of <i>Vibrio parahaemolyticus</i> and total viable bacteria count viability in the Pacific oyster ( <i>Crassostrea gigas</i> ) .....	66
2.1 Introduction .....	66
2.2 Materials and Methods .....	68
2.3 Results .....	77
2.4 Discussion .....	86
3 – Evaluation of a software program for predicting <i>Vibrio parahaemolyticus</i> and total viable bacteria count levels in Pacific oysters ( <i>Crassostrea gigas</i> ) in simulated supply chain studies .....	95
3.1 Introduction .....	95
3.2 Materials and Methods .....	97
3.3. Results .....	103
3.4 Discussion .....	110
4 – A cold chain management tool for the safety and quality of live oysters: a case study .....	114
4.1 Introduction .....	114
4.2 Materials and Methods .....	116
4.3 Results .....	133
4.4 Discussion .....	139
5 - Influence of storage temperature on bacterial communities in live Pacific oysters ( <i>Crassostrea gigas</i> ) .....	148
5.1 Introduction .....	148
5.2 Materials and Methods .....	150

5.3 Results .....	155
5.4 Discussion .....	168
6 – Conclusions and future work .....	175
6.1 Introduction .....	175
6.2 Findings .....	176
6.3 Future .....	179
List of references .....	183
Appendix A - <i>Vibrio parahaemolyticus</i> in <i>C. gigas</i> temperature model .....	204
A1. Oyster injection test.....	204
A2. TCBS and conventional MPN comparison for <i>Vibrio parahaemolyticus</i> enumeration .....	205
A3. <i>V. parahaemolyticus</i> and TVC secondary model data analysis .....	206
Appendix B – Cold chain management tool for oysters: a case study .....	207
B1. Sensitivity analyses for the simulation .....	207
Appendix C - Storage temperature effects on bacterial communities in <i>C. gigas</i> .....	209
C1. Clone libraries analysis in fresh and stored Pacific oysters.....	209
C2. TRFLP profiles for clones .....	212



## List of abbreviations

APW	Alkaline Peptone Water
ANOSIM	Analysis of Similarity
ASQAP	Australian Shellfish Quality Assurance Program
BOM	Bureau of Meteorology
CDC	Centre for Disease Control and prevention
CFU	Colony-Forming Unit
DPI	Department of Primary Industries
FAO	Food and Agriculture Organization
HPP	High-Pressure Processing
MA	Marine Agar
mAPW	modified Alkaline Peptone Water
MB	Marine Broth
MDS	Non-metric Multidimensional Scaling
MPD	Maximum Population Density
MPN	Most Probable Number
MPRM	Modular Process Risk Model
mTSA	modified Tryptone Soy Agar
mTSB	modified Tryptone Soy Broth
PCR	Polymerase Chain Reaction
PO	Pacific Oyster
PSS	Peptone Salt Solution
QMRA	Quantitative Microbial Risk Assessment

RFID	Radio Frequency Identification Device
RFU	Relative Fluorescent Unit
RMSE	Root Mean Square Error
SARDI	South Australian Research and Development Institute
SD	Standard Deviation
SRO	Sydney Rock Oyster
SSO	Specific Spoilage Organisms
SSSP	Seafood Spoilage and Safety Predictor
TAE	Tris-Acetate-EDTA
TCBS	Thiosulfate Citrate Bile Salts
TDH	Thermostable Direct Hemolysin
TLH	Thermolabile Hemolysin
TRH	Thermostable Related Hemolysin
TTI	Time-Temperature Integrators
TVC	Total Viable bacteria Count
USFDA	U.S. Food and Drug Administration
USNSSP	U.S. National Shellfish Sanitation Program
VQRA	Quantitative Risk Assessment on the public health impact of pathogenic <i>Vibrio parahaemolyticus</i> in raw oysters
WHO	World Health Organisation

## List of tables

<b>Table 1.</b> Oyster production, exports and imports for 2007-08 by state in Australia .....	25
<b>Table 2.</b> Major pathogenic bacteria associated with seafood classified by habitat. ....	32
<b>Table 3.</b> Optimum and growth range conditions for <i>V. cholerae</i> , <i>V. vulnificus</i> and <i>V. parahaemolyticus</i> .....	33
<b>Table 4.</b> A summary for long-term studies in the incidence of <i>V. parahaemolyticus</i> in oysters .....	39
<b>Table 5.</b> A summary of secondary models for <i>V. parahaemolyticus</i> .....	58
<b>Table 6.</b> Predictive microbiology applications .....	59
<b>Table 7.</b> Harvest conditions during oyster collection (oyster batches used during model development).....	77
<b>Table 8.</b> Harvest conditions during oyster collection (oyster batches used during model evaluation).....	78
<b>Table 9.</b> Secondary models for <i>V. parahaemolyticus</i> and total viable bacteria count in Pacific oysters .....	100
<b>Table 10.</b> Change in <i>V. parahaemolyticus</i> levels for the long refrigerated supply chain .....	105
<b>Table 11.</b> Change in total viable bacteria count for the long refrigerated supply chain ...	106
<b>Table 12.</b> Change in <i>V. parahaemolyticus</i> levels for the short refrigerated supply chain .....	106
<b>Table 13.</b> Change in total viable bacteria levels for the short refrigerated supply chain .....	107
<b>Table 14.</b> Change in <i>V. parahaemolyticus</i> levels for the short non-refrigerated supply chain .....	107

<b>Table 15.</b> Change in total viable bacteria count for the short non-refrigerated supply chain .....	108
<b>Table 16.</b> Summary of the oyster supply chain profiles for Tasmania .....	119
<b>Table 17.</b> Input data and distributions used in the case study for the short supply chain .....	122
<b>Table 18.</b> Input data and distributions used in the case study for the long supply chain .....	123
<b>Table 19.</b> Summary rank (higher:1 to lower:5) for tornado plots analyses of model inputs for <i>V. parahaemolyticus</i> levels at consumer storage. ....	138
<b>Table 20.</b> Summary rank (higher:1 to lower:5) for tornado plots analyses of model inputs for total viable bacteria count levels at consumer storage.....	139
<b>Table 21.</b> Total viable bacteria count in different Pacific oyster batches depending on storage temperature .....	156
<b>Table 22.</b> Statistic significance (P-value) of differences between bacterial clone communities calculated based on partial sequences of 16S rRNA .....	158
<b>Table 23.</b> Good's coverage values and representative genera in each clone library for six different oyster samples.....	161

## List of figures

<b>Figure 1.</b> Pictures for Sydney Rock (left) and Pacific (right) oysters. ....	26
<b>Figure 2.</b> Agarose (2%) gel electrophoresis of <i>V. parahaemolyticus</i> PCR products corresponding to <i>tlh</i> (~200bp), <i>tdh</i> (~250bp) and <i>trh</i> (~500bp) genes. ....	79
<b>Figure 3.</b> Agarose (2%) gel electrophoresis of <i>V. parahaemolyticus</i> PCR products corresponding to <i>tlh</i> gene (~200bp) in oyster enrichment samples inoculated with serial 10-fold dilutions of the <i>V. parahaemolyticus</i> cocktail. ....	79
<b>Figure 4.</b> Growth of <i>V. parahaemolyticus</i> in live Pacific oysters (18.4 - 30.4°C). ....	80
<b>Figure 5.</b> Inactivation of <i>V. parahaemolyticus</i> in live Pacific oysters (3.6 - 12.6°C). ....	81
<b>Figure 6.</b> Secondary models for <i>V. parahaemolyticus</i> : growth (a) and inactivation (b). ....	82
<b>Figure 7.</b> Comparison of different secondary models for <i>V. parahaemolyticus</i> growth. ....	83
<b>Figure 8.</b> Growth of total viable bacteria count in live Pacific oysters (3.6 - 30.4°C). ....	84
<b>Figure 9.</b> Secondary model for total viable bacteria count (a) and its comparison to <i>V. parahaemolyticus</i> secondary models (b). ....	86
<b>Figure 10.</b> Oyster harvest sites. ....	98
<b>Figure 11.</b> Example of data input for the tertiary model. ....	101
<b>Figure 12.</b> Example of outputs from the tertiary model. ....	102
<b>Figure 13.</b> Temperature profiles for simulated supply chains. ....	104
<b>Figure 14.</b> Comparison of observed versus predicted growth rates for <i>V. parahaemolyticus</i> (up) and total viable bacteria count (down). ....	109

<b>Figure 15.</b> Plots of the ratio between predicted and observed growth rates for <i>V. parahaemolyticus</i> (left) and total viable bacteria count (right) based on supply chain scenario and site. ....	110
<b>Figure 16.</b> Structure for the long oyster supply chain from harvest to consumer storage. ....	117
<b>Figure 17.</b> Structure for the short oyster supply chain from harvest to consumer storage. ....	118
<b>Figure 18.</b> Normal probability density distribution for seawater temperature at selected harvest areas in Tasmania for summer (right) and winter (left). ....	124
<b>Figure 19.</b> Normal probability density distribution for total viable bacteria count in Pacific oysters after harvest in Tasmania and after shipment from New South Wales. ...	125
<b>Figure 20.</b> Comparison of Pert and normal probability density distribution for storage temperature at depot in summer. ....	129
<b>Figure 21.</b> Comparison of Pert and normal probability density distribution for storage temperature at consumer. ....	130
<b>Figure 22.</b> Weibull probability distribution for times for consumer or retail transport. ..	132
<b>Figure 23.</b> Predicted levels of total <i>V. parahaemolyticus</i> in oysters after consumer storage in the long supply chain in summer (left) and winter (right). ....	134
<b>Figure 24.</b> Predicted levels of total <i>V. parahaemolyticus</i> in oysters after consumer storage in the short supply chain in summer (left) and winter (right). ....	135
<b>Figure 25.</b> Predicted total viable bacteria counts levels in oysters after consumer storage in the long supply chain in summer (left) and winter (right). ....	136
<b>Figure 26.</b> Predicted total viable bacteria counts levels in oysters after consumer storage in the short supply chain in summer (left) and winter (right). ....	137
<b>Figure 27.</b> Spider plot showing the influence of seawater temperature in the predicted mean levels of <i>V. parahaemolyticus</i> after consumer storage for the long (left) and the short (right) supply chain in summer. ....	138
<b>Figure 28.</b> Temperature abuse what-if scenario: predicted levels of total <i>V. parahaemolyticus</i> in oysters prior to consumption in summer for the long (left) and the short (right) supply chains. ....	145

<b>Figure 29.</b> Multidimensional scaling plot based on Bray-Curtis similarities of TRFLP data. ....	157
<b>Figure 30.</b> Cluster analysis of the six different clone library compositions obtained by Fast UniFrac. ....	159
<b>Figure 31.</b> Clone library composition of bacteria in homogenates of six batches of Pacific oysters. ....	160
<b>Figure 32.</b> 16S rRNA phylogenetic tree of representative genera for the phylum <i>Bacteroidetes</i> from sample clone libraries (bold letters) and type strains, based on the Kimura 2-parameter model and the neighbor-joining method. ....	163
<b>Figure 33.</b> 16S rRNA phylogenetic tree of representative genera for the phylum <i>Proteobacteria</i> from sample clone libraries (bold letters) and type strains, based on the Kimura 2-parameter model and the neighbor-joining method. ....	164
<b>Figure 34.</b> 16S rRNA phylogenetic tree of representative genera for phyla <i>Spirochaetes</i> , <i>Fusobacteria</i> , <i>Cyanobacteria</i> , <i>Tenericutes</i> and <i>Planctomycetes</i> from sample clone libraries (bold letters) and type strains, based on the Kimura 2-parameter model and the neighbor-joining method. ....	165
<b>Figure 35.</b> Terminal restriction fragment length profiles for six different oyster samples. Identification of fragments as <i>Psychrilyobacter</i> (PSY) and <i>Alkaliflexus</i> -related (ALK-R). ....	167

# **1 – Introduction**

This first chapter provides brief background information about the oyster industry in Australia, safety and quality issues in oysters, supply chain management and predictive microbiology to the reader. The content includes recent studies in the area and identifies some data gaps that this thesis is oriented to fill.

## **1.1 Oyster industry in Australia**

Oyster farming is one of the oldest aquaculture industries in Australia. Oyster cultivation began in New South Wales around 1870 when oyster farmers introduced sticks, stones, and shells to catch and grow oysters in the intertidal zone until they could be harvested (18).

Nowadays, oyster farming is an important economic seafood sector. Statistic data for the period 2007-08 show that Australia produced 12,460 tonnes of edible oysters with a value of \$89.1 million Australian dollars at the farm gate (Table 1). The major production state was South Australia followed by New South Wales and Tasmania. A total of 228 tonnes were exported and 726 tonnes imported. From the imported oysters, an approximate 99% came from New Zealand (5).



**Table 1.** Oyster production, exports and imports for 2007-08 by state in Australia

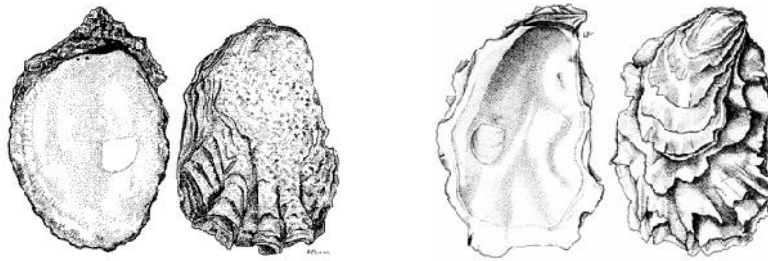
	New South Wales	South Australia	Tasmania	Other states	Total
<b>Value AUS'000</b>	39,000	30,132	19,378	620	89,130
<b>Quantity (tonnes)</b>	4,500	5,448	2,512	0	12,460
<b>Production (%)</b>	36	44	20	0	100
<b>Exports (tonnes)</b>	8	139	66	8	228
<b>Imports (tonnes)</b>	-	-	-	-	726

Source data: Anonymous, 2009 (5).

### Oyster culture

Oysters belong to the class *Bivalvia* and the phylum *Mollusca*. The class *Bivalvia* consists of at least 7500 species including other animals with two shell valves such as clams, mussels and cockles (88). Edible oysters classify in the family *Ostreidae* and are primarily of the genera *Ostrea*, *Crassostrea*, *Saccostrea*, and *Ostreola*.

The two principal oyster species grown in Australia are the native Sydney Rock Oyster (SRO, *Saccostrea glomerata*) and the introduced Pacific Oyster (PO, *Crassostrea gigas*) (Figure 1). SRO account for around 40% of the total Australian production of oysters, with PO at 60%. The native flat (*Ostrea angasi*), the black-lip (*Saccostrea echinata*) and the milky (*Saccostrea amasa*) oysters are only semi-commercially produced and represent less than 1% of the total industry supply (47).



Source pictures : various authors (7, 18).

**Figure 1.** Pictures for Sydney Rock (left) and Pacific (right) oysters.

The main oyster species produced commercially in Tasmania is the PO, which was introduced from Japan in 1947-52. A decrease in wild oyster production observed in 1970s gave way to the development of hatcheries. Available spat (the name for larval oysters) was provided to South Australian farmers allowing the establishment of a viable PO industry. In New South Wales, PO are only grown in specific areas and they are treated as a pest due to its interaction with the native SRO, which is the main species cultured in this location (134).

SRO and PO present differences in salinity and temperatures tolerance during harvest. SRO is essentially subtropical in character and the salinity tolerance range is reported to be 1.5 - 5.0%, with an optimum between 2.5 - 3.5%. PO can survive higher salinities of 5.5% and presents a broader temperature tolerance, ranging from  $-1.8$  to  $35^{\circ}\text{C}$ , allowing its culture in high salinity and lower temperature estuaries (7, 165).

### Oyster consumption

The low percentage of export and imports (Table 1) shows that most of the oysters consumed in Australia are produced locally.

Oysters may be purchased fresh (half shell or alive), frozen (removed from the shell), cooked, bottled or canned. When alive, they present closed shells. A 2003 consumer survey revealed fresh oysters were preferred by 73.3% of Australians consumers (234). In addition, it was observed that the greatest proportion of oyster consumers was in the 40 - 59 year old range and that 15 - 19 year age range are the least likely to consume oysters. From the consumers who declared they eat oysters, the frequency of oyster consumption for 41.7% was less than four times per year and only 18.4% consumed oysters more than 10 times a year. The same study identified quality as the most important factor considered by oyster purchasers, and health concerns as one of the barriers to oyster consumption.

The type of oyster purchased, the consumption style and the cooking method are important factors for the risk of illness and shelf-life of the product. In the case of pathogenic bacteria, it has been observed that the risk of *Vibrio parahaemolyticus* illness increases for raw oyster eating population (17, 23, 119, 130), and susceptible individuals are encouraged to cook the product in order to eliminate bacteria (17). However, typical cooking will not eliminate all the biological risks associated with oysters.

Viruses are also a frequent cause of seafood-related infections. Viruses differ to bacteria in resisting usual cooking temperatures as well as in surviving inside the shellfish independently to the oyster storage temperature (124). The required time and temperature to inactivate viruses (e.g. 90°C for 1.5 min based on data obtained for hepatitis A in

cockles) can result in an unpalatable product and the processes of steaming and freezing are not able to completely eliminate their presence (124, 180). Outbreaks due to viruses have been associated with raw, half shell, frozen oyster meat and cooked oysters (12, 14, 226, 228).

Studies comparing shell-stock (alive) and shucked (half shell) oysters have shown that *V. parahaemolyticus* can survive in both forms of the product but reduction during cold storage is higher in shucked product (205). However, a significantly higher total bacteria load has been observed in shucked in comparison to shell-stock (104) suggesting a shorter shelf-life for this type. Moreover, shucked oysters are more exposed to cross-contamination during handling and storage.

### Regulation

Bivalves can reduce or remove contaminants when transferred to clean natural harvest waters (relaying) or under controlled conditions in tanks (depuration). During depuration, oysters are placed in chemically (e.g. chlorine, ozone) or physically (e.g. UV irradiation) disinfected seawater tanks, typically for 24 to 48 h (172).

Oyster depuration was first developed in the 1920s as a result of the increase of shellfish-associated illnesses (i.e. cholera and typhoid fever) (188). Although depuration has been shown to be effective for reducing *E. coli*, it is not an adequate intervention to decrease other biological hazards present in oysters, such as *V. parahaemolyticus* or viruses (52, 124).

Oyster depuration technology was introduced in New South Wales in the 1970s after the occurrence of gastroenteritis cases linked to oyster consumption in the region. However, several episodes of gastroenteritis caused by Norovirus ten years later and a major outbreak of gastroenteritis linked to Hepatitis A in 1997 were associated with oysters (48, 93). As a response, a shellfish quality assurance program was formally implemented that year. In contrast to New South Wales, the coastlines in Tasmania and South Australia are not very heavily urbanised and depuration systems are not used (134).

The Australian Shellfish Quality Assurance Program (ASQAP) follows the internationally accepted United States National Shellfish Sanitation Program (USNSSP) (4, 20). The main objective is to limit the oyster farming to harvest water shown to be exempt from harmful contaminants and pathogenic micro-organisms. Each harvest area completes a sanitary survey to classify it as approved, approved conditional, restricted or prohibited. The ASQAP provides the requirements for completing and maintaining sanitary surveys, and the management of growing areas (4).

The ASQAP uses a limit of either a total or faecal coliform standard as a bacteriological water-quality standard. The median faecal coliform MPN value for approved growing areas must not exceed 14 per 100 ml of sample, with no more than 10% of the samples exceeding an MPN value of 43 for a five-tube, or 49 per 100 ml for a three-tube decimal dilution test (4). Although the presence of sewage may add nutrients to the water and would enhance survival of some bacterial species, it is well documented that the environmental presence of *V. parahaemolyticus* does not distinctly correlate with the presence of human enteric bacteria (60, 61, 176).

Regarding the examination of shellfish, the Australia New Zealand Food Standards Code 1.6.1 contains information about the maximum permissible levels for *Staphylococcus*, *Salmonella*, standard plate count and *E. coli*. At the moment, there is no regulation for *Vibrio* spp. in Australia. A recommendation level for *V. parahaemolyticus* of  $\leq 10,000$  cells/g was proposed in the USA (17). The European Union has concluded that controlling exclusively total levels may not be appropriate as there is a lack of correlation between total and pathogenic *V. parahaemolyticus* levels (13). The Codex Alimentarius elaborated a risk management document “Guidelines on the application of general principles on food hygiene for the control of pathogenic *Vibrio* spp. in seafood” which is being updated to assist its implementation in different regions and countries (10, 11).

The ASQAP also includes controls during the post-harvest processing (depuration and relaying), handling, storage, transport and identification of shellfish. A temperature control is required, and shellfish intended for consumption as raw product must be placed under ambient refrigeration at  $\leq 10^{\circ}\text{C}$  within 24 h of harvest, with the exception of SRO which are allowed to be stored no warmer than  $25^{\circ}\text{C}$  for the first 72 h post-harvest and no warmer than  $15^{\circ}\text{C}$  thereafter (4).

Temperature control is also a requirement under export regulations. In Australia, the Export Control (Fish and Fish Products) Orders 2005 has a shipping temperature requirement of  $\leq 5^{\circ}\text{C}$ . This low temperature may be too low for the storage of live product and could kill and thereby decrease the quality of the product. The effect of cold stress resulting in mortality has been observed during oyster quality studies (1).

## 1.2 Microbiological safety of oysters

Bivalves represent a special case among the microbial hazards associated with food.

Oysters filter large volumes of water (up to 4 litres per hour (190)) across their gills to obtain oxygen and food. Food particles are trapped on the gills and transported toward the mouth by specialized cilia, the small food particles are then transported to the digestive gland (49). Any pathogenic micro-organism present in the harvest site can be concentrated in the animal. Accumulated harmful micro-organisms can become a hazard when the whole oyster including the viscera is consumed raw or only lightly cooked.

Epidemiology data show that enteric viruses are the most common pathogen transmitted by bivalves. Hepatitis A is one of the most serious illnesses associated to shellfish causing a serious debilitating disease and even, occasionally, death. However, the most frequently reported illnesses are caused by Norovirus. In Australia, oyster associated outbreaks with Hepatitis A and Norovirus have been reported (48, 77, 226).

Among the different pathogenic bacteria which are indigenous to estuaries (Table 2) and naturally present in oysters, marine *Vibrio* spp. is the leading bacterial pathogen involved in shellfish-associated outbreaks (180). Other bacteria that have been occasionally implicated in gastroenteritis and linked to shellfish are usually due to sewage pollution: *Salmonella* spp., *Shigella* spp., *Listeria* spp and *E. coli* (166, 180).

**Table 2.** Major pathogenic bacteria associated with seafood classified by habitat.

<b>Major pathogenic bacteria associated with seafood</b>	
Indigenous	<i>Clostridium botulinum</i> type E, B, F; <i>Vibrio cholerae</i> ; <i>Vibrio parahaemolyticus</i> ; <i>Vibrio vulnificus</i> ; <i>Aeromonas hydrophila</i> ; <i>Plesiomonas shigelloides</i> .
External (primary habitat)	<i>Clostridium botulinum</i> type A and B (soil); <i>Listeria monocytogenes</i> (soil, foilage, faeces, seafood, processing environments); <i>Shigella</i> spp (faecal contamination); <i>Salmonella</i> spp (faecal contamination); <i>Staphylococcus aureus</i> (pond water, human carrier); <i>Escherichia coli</i> (faecal contamination).

Source data: various authors (89, 166).

### 1.2.1 The genus *Vibrio*

The genus *Vibrio* is classified in the family *Vibrionaceae*, a member of the class *Gammaproteobacteria*. *Vibrio* spp. are Gram negative, non-spore-forming, facultative anaerobic rods, which are often curved in shape and usually motile by a single polar flagellum (64).

Only 12 *Vibrio* spp. are known to be associated with human infections (114) among the 81 recognized species (<http://www.bacterio.cict.fr/uw/vibrio.html>, [accessed 30/05/11]).

*Vibrio parahaemolyticus*, *V. cholerae*, and *V. vulnificus* are the principal *Vibrio* spp. linked to seafood-borne infections. *V. cholerae* causes human cholera and was first described in 1854 by Pacini (32). *V. parahaemolyticus* was first identified in 1950 as the cause of



gastroenteritis from the consumption of contaminated dried sardines in Japan (81).

*V. vulnificus* was first isolated from a wound infection and described in 1979 (76).

The growth of *Vibrio* spp. is stimulated by the presence of sodium ions and some species have a requirement for salt for growth (Table 3). *V. cholerae* is able to grow without added salt although their growth is stimulated by the presence of sodium ions. In contrast, *V. parahaemolyticus* and *V. vulnificus* require the addition of 2.5 - 3% NaCl to culture media for optimum growth (64).

**Table 3.** Optimum and growth range conditions for *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*

	Temperature		NaCl (%)		pH		Water activity	
	Range	Optimum	Range	Optimum	Range	Optimum	Range	Optimum
<i>V. cholerae</i>	10-43	37	0.1-4.0	0.5	5.0-9.6	7.6	0.970-0.988	0.984
<i>V. vulnificus</i>	8-43	37	0.5-5.0	2.5	5.0-10.0	7.8	0.960-0.997	0.980
<i>V. parahaemolyticus</i>	5-43	37	0.5-10	3.0	4.8-11.0	7.8	0.940-0.996	0.981

Source data: Desmarchelier 2003 (64).

The three major clinical manifestation of *Vibrio*-associated disease are: wound infection, primary septicemia and gastroenteritis (71). From the pathogenic *Vibrio* spp.,

*V. parahaemolyticus* is the most associated with gastroenteritis. In fact,

*V. parahaemolyticus* is the leading seafood-borne disease outbreaks in Taiwan, Japan (71) and the leading cause of *Vibrio*-associated gastroenteritis in the USA (180).

*Vibrio cholerae* serotypes 01 and 0139 cause cholera, a disease with epidemic and pandemic potential. *Vibrio cholerae* serotypes other than 01 and 0139 can cause moderate

gastroenteritis and are associated with sporadic cases and small outbreaks (74). Cholera symptoms include vomiting and characteristic watery diarrhoea. If not treated appropriately, the electrolyte imbalance due to dehydration can result in tachycardia, hypotension and vascular collapse. The fluid loss can be so pronounced that an infected person can die within hours (74, 114).

*Vibrio vulnificus* infections are rare and generally limited to susceptible populations (e.g. chronic liver disease, immunosuppressive disorders). However, *V. vulnificus* presents the highest case-fatality rate (approximately 50%) of the shellfish-borne infections among vulnerable population (75, 114).

### **1.2.2 *Vibrio parahaemolyticus***

#### Pathogenesis

Most clinical isolates of *V. parahaemolyticus* from patients with diarrhoea produce an enzyme that can lyse blood cells on Wagatsuma blood agar plates (102, 199). Its production is termed the Kanagawa phenomenon and the strain is often reported as “KP-positive”. The Kanagawa reaction is caused by a protein named Thermostable Direct Hemolysin (TDH), indicating it is not inactivated by heat (100°C for 10 min) and that its direct haemolytic activity on erythrocytes is not enhanced by the addition of lecithin (71). TDH has multiple biological activities, including hemolysis, enterotoxicity, cytotoxicity, and cardiotoxicity (101, 156, 183). A second hemolysin called the Thermostable Related Hemolysin (TRH) was also identified after an outbreak in 1988 in travellers who visited

the Republic of Maldives (100, 103, 168). TDH and TRH are immunologically and biologically similar; the nucleotide sequences of the genes encoding both proteins show approximately 70% similarity. However, TRH is less thermostable than TDH and inactivates at 60°C for 10 min (232).

Although TDH and TRH are major virulence factors, others have been reported (222). The capacity of adhesion to human intestinal cells, extracellular enzymes and cell wall components are thought to be involved in pathogenicity (64, 71, 173). A heat-labile protein (protease A) produced by a clinical *V. parahaemolyticus* strain *tdh*- and *trh*- has been identified as a potential virulent factor. Protease A presents haemolytic and cytotoxic activity (123). Furthermore, recent outbreaks have been related to a non-pandemic *tdh*- and *trh*- strain (82).

Gastroenteritis caused by *V. parahaemolyticus* is almost exclusively associated with seafood which is consumed raw, inadequately cooked, or cooked but re-contaminated (173). The incubation period is usually between 8 to 72 h and illness includes acute diarrhoea and abdominal pain for up to 72 h (114). Other symptoms, reported less frequently are nausea, vomiting, headache, low-grade fever and chills. The organism causes damage to the gut mucosa and colonic ulceration. There is a low rate of mortality and death usually occurring only in the case of elderly or debilitated patients. Severe cases of gastroenteritis may require hospitalization, although most are treatable with supportive therapy such as rehydration. Treatment with tetracycline has proven beneficial in cases of prolonged infection (114). In rare cases, wound infection and septicaemia is also possible (53, 177). Studies using human volunteers in Japan showed that ingestion of  $2 \times 10^5$  to  $3 \times 10^7$  cells of the TDH-positive strains can result in gastrointestinal disease (173).

## Epidemiology

A high incidence of *V. parahaemolyticus* food poisoning is reported in the USA (58, 227). Outbreaks and sporadic cases have been reported since the first confirmed 425 cases associated with consumption of steamed crabs in Maryland in 1971. Two major outbreaks associated with consumption of raw oysters occurred in the Pacific Northwest in 1997 and in Texas in 1998 involving 209 and 416 cases, respectively (17). In addition, recent data from the USA Centre for Disease Control and prevention (CDC) indicate an increase in *Vibrio* spp. infections from 2001 to 2008, and that *V. parahaemolyticus* is responsible for 52% of the cases of *Vibrio* infections confirmed in 2009 (16).

However, *V. parahaemolyticus* disease occurs worldwide (209). In Asia, *V. parahaemolyticus* is a common cause of seafood-borne illness. Since the first *V. parahaemolyticus* outbreak reported in 1951, other incidents have been reported in Japan, Taiwan and China (208). In Europe, *V. parahaemolyticus* infections occurred in France and Spain. Two major outbreaks reported in Spain include 64 cases associated with raw oysters in 1999 and 80 illnesses after eating boiled crab in 2004 (142). More recently a high incidence in *V. parahaemolyticus* illnesses was experienced in Chile, where approximately 7,000 cases were reported during 2004 to 2007 (98).

In Australia, *V. parahaemolyticus* illnesses have been reported in New South Wales. An outbreak associated with seafood was observed between 1977 and 1984 involving 60 cases (59). Three different outbreaks associated with cooked prawns from Indonesia involved more than 100 cases and one death in 1990, and more than 50 cases in 1992 (119). In 1992,

a death was associated with the consumption of raw oysters (119). In 2005, two people were reported sick after consumption of prawns and oysters (6).

### Environmental incidence

*Vibrio* spp. are naturally occurring environmental bacteria, present worldwide in coastal waters. The effect of salt composition influences their distribution in aquatic systems (64, 114). Waters with lower salinity ranges (i.e. 0.2 - 0.5%) favour *V. cholerae* and *V. vulnificus*, while these species are recovered less frequently from waters with salinity above 3% (114).

The geographical distribution and incidence of *Vibrio* spp. in the natural environment within a specific area are determined by multiple interacting factors, including:

- Water temperature is positively correlated to total *V. parahaemolyticus* levels (61, 63, 176) with exception of tropical coastal regions where temperature is always optimal for its recovery (60). The presence of *V. parahaemolyticus* is detected when water temperature is greater than 14 - 15°C (111, 176). This is related to a seasonal incidence of *Vibrio* spp., with a higher level in the warmer months (61, 176).
- Salinity is significantly associated with environmental *V. parahaemolyticus* levels in some situations (63, 111) while not in others (60, 61, 176). Differences among studies can be a consequence of the range of variation of salinity studied, as it may be more likely to find significance when a wider salinity range is studied.

- A correlation has been observed between turbidity, chlorophyll a and dissolved oxygen and *V. parahaemolyticus* levels, while a lack of significant association has been found for pH (111, 176).
- The relationships between environmental factors have been tested more extensively for *V. parahaemolyticus* at the species level than for pathogenic strains, partly due to the lack of available sensitive technologies, until recently. However, differences between total and pathogenic *V. parahaemolyticus* levels, and influential environmental factors for predicting abundances, have been observed. For example, turbidity appears to be the most influencing factor for pathogenic strains, while temperature is more influential on total *V. parahaemolyticus* (111).

Due to the halophilic nature of *V. parahaemolyticus*, raw seafood can be naturally contaminated. It has been observed that oysters accumulate *V. parahaemolyticus* via filter-feeding of seawater to concentrations 100 times greater than those found in the surrounding seawater (61). Where environmental conditions are favourable, typical levels of total *V. parahaemolyticus* in oysters may be 2 - 3 log<sub>10</sub> Colony-Forming Unit (CFU)/g (61, 111, 176) and in some cases reach levels higher than 10<sup>4</sup> CFU/g in warmer months (60, 63). Pathogenic levels of *V. parahaemolyticus* are typically several logs lower than total *V. parahaemolyticus* (62, 238). The reported frequency of *tdh* detection in oysters for studies of at least one year duration report ranges from 3 - 70% (60, 63, 111, 176) and 17-60% for *trh* (111) depending on the sensitivity of the methodology and the region studied (Table 4).

**Table 4.** A summary for long-term studies in the incidence of *V. parahaemolyticus* in oysters

	Study description	Total <i>V. parahaemolyticus</i> after harvest				Significant association with <i>V. parahaemolyticus</i>		Pathogenic <i>V. parahaemolyticus</i> after harvest			
	Oyster specie	Period (years)	Range (log <sub>10</sub> )	Mean (log <sub>10</sub> )	Samples (%)	Yes	No	TDH mean (log <sub>10</sub> )	Samples (%)	TRH mean (log <sub>10</sub> )	Samples (%)
<i>DePaola et al. 1990</i>	<i>Crassostrea virginica</i>	1	-	max 2.2	-	T	S	-	-	-	-
<i>DePaola et al. 2003</i>	<i>Crassostrea virginica</i>	1.5	<1-4.1 <sup>ci</sup>	-	-	T,S	-	0.1,0.4	12.8 <sup>ci</sup>	-	-
<i>Deepanjali et al. 2005</i>	<i>Crassostrea madrasensis</i>	1	<1-4 <sup>ci</sup>	~3.5 <sup>ci</sup>	93.9 <sup>ci</sup>	-	T,S	max 2.1 <sup>ci</sup>	10.2 <sup>ci</sup> ,6.1 <sup>pc</sup>	-	~60 <sup>pc</sup>
<i>Parveen et al. 2008</i>	<i>Crassostrea virginica</i>	1	<1-2.77 <sup>ci</sup>	~ 2 <sup>ci</sup>	79 <sup>ci</sup>	T,t,D	S,P,C	1	3 <sup>ci</sup>	-	-
<i>Johnson et al. 2010</i>	<i>Crassostrea virginica</i>	1.4	<1-3.4 <sup>ci</sup>	~ 2.4 <sup>ci</sup>	87 <sup>ci</sup> ,94 <sup>rt</sup>	T,S,t	-	-0.9	20 <sup>ci</sup> ,70 <sup>rt</sup>	-1.5	17 <sup>ci</sup> ,60 <sup>rt</sup>

ci: colony hybridization, pc: MPN and conventional PCR, rt: MPN and real-time PCR, max: maximum level, T: temperature, S: salinity, P: pH, C: chlorophyll a, t: turbidity, D: dissolved oxygen. Source data: various authors (60, 61, 63, 111, 176).

In Australia, there has not been a long-term study of the incidence of *V. parahaemolyticus* but it has been isolated from oysters in several studies (65, 72, 73, 125, 133). Using colony hybridization method, total *V. parahaemolyticus* was detected in 80% of New South Wales, 60% of Tasmanian and 20% of South Australian oysters in autumn months, at average levels of 2.4 to 3.0 log<sub>10</sub> CFU/g. In the same study, pathogenic *V. parahaemolyticus* *tdh*<sup>+</sup> were found in 20% of New South Wales and Tasmanian, and in 10% of South Australian oysters at average levels of 2.0 to 2.4 log<sub>10</sub> CFU/g (125).

Climate change impacts seawater and surface air temperatures, precipitation and stream flow patterns. These changes can occur over relatively short periods of times and areas during different seasons, influencing proliferation and changes in the distribution of micro-organisms in regions (138, 140). An example of *V. parahaemolyticus* illnesses due to climate anomalies is the outbreak in coastal Peru which were subsequently linked to the 1997 El Niño episode (141).

### Identification

The most common and conventional method for routine analysis of *V. parahaemolyticus* includes a selective enrichment in Alkaline Peptone Water (APW) followed by plating of the enrichment on Thiosulfate Citrate Bile Salts (TCBS) agar. This method in combination with biochemical identification is recommended as standard test (USFDA BAM, AS/NZS 1766.2.9:1997, ISO/TS 21872-1:2007)(115). Although highly selective, a limitation to the use of TCBS is the time and labour for confirmation of colonies, as the media does not



readily differentiate *Vibrio* spp., other than sucrose- and non-sucrose-fermentation. A chromogenic agar able to differentiate *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* with respect to other *Vibrio* spp. is commercially available (CHROMagar™ Vibrio, Dutech Diagnostics, NSW, Australia). This agar medium is based on the detection of beta-galactosidase activity to differentiate *V. parahaemolyticus* using a chromogenic substrate (97). The use of CHROMagar™ Vibrio has been shown to be more accurate and specific than TCBS for the isolation of *V. parahaemolyticus* in shellfish (69).

For enumeration purposes, a Most Probable Number (MPN) method is used during enrichment. The MPN analysis coupled with plating is laborious and can take seven to ten days. The combination of MPN with a species-specific Polymerase Chain Reaction (PCR) method enables the completion of enumeration within two days. This MPN-PCR format has been used for enumeration of pathogenic *V. parahaemolyticus* (219). In molecular tests, the *tlh* (thermolabile hemolysin) gene is used as a species-specific marker (213) while the *tdh* (167) and the *trh* (168) genes are pathogenicity markers. Other molecular methods used to enumerate *V. parahaemolyticus* in shellfish include colony hybridization and real-time PCR (85, 169).

### Control measures

In order to reduce the presence of *V. parahaemolyticus* in oysters, different post-harvest operations have been studied. Depuration has limited effects on the elimination of *V. parahaemolyticus* in oysters (72) and the level of reduction is influenced by the temperature of the operation (44). Heat treatment of 50°C during 10 min has been shown to

reduce *V. parahaemolyticus* from  $10^5$  MPN/g to non-detectable levels, but the treatment may cause changes in the texture due to protein denaturation (3). High-Pressure Processing (HPP) is a non-thermal method used without apparent changes in oyster nutrients, flavour and appearance. A HPP treatment of 293 MPa for 2 min can achieve greater than 3.52  $\log_{10}$  reduction of *V. parahaemolyticus* in oysters (131). Similar to heat treatment, HPP will destroy the adductor muscle and oysters need to be banded to prevent opening shell during treatment (209). The main disadvantage is the high cost of initial investment in HPP equipment. Another effective means for eliminating *V. parahaemolyticus* in oysters without causing changes in the texture or sensory properties is irradiation (135). However, the reluctance among consumers to accept irradiated food and the need to safely handle radioactive material has limited its use.

Freezing has also been demonstrated to reduce levels of *V. parahaemolyticus* in oysters and its reduction depends on the time of storage. A 0.22  $\log_{10}$  reduction was observed in inoculated oysters after an ultra-low flash-freezing process (-95.5°C for 12 min). A subsequent storage of frozen oysters at -10°C for one and six months resulted in a 2.45 and 4.55  $\log_{10}$  *V. parahaemolyticus* reduction, respectively (126). The use of prolonged refrigeration has also been shown to reduce levels of *Vibrio* spp. in oysters. Storage of *V. parahaemolyticus*-inoculated oysters at 5°C for 96 h showed a reduction of 1.42  $\log_{10}$  (205). However, it has been observed that *V. parahaemolyticus* can survive in oysters stored at 4°C for 3 weeks indicating that refrigeration may not eliminate it completely (112).

### 1.3 Microbiological quality of oysters

Changes in oyster quality during storage have been assessed by sensory, chemical and microbiological analyses (1, 24). Sensory analysis usually includes information about product odour, appearance, texture and taste. The freshness of oyster is determined by the absence of odours such as dry sea-weed, ammonia and mud, and the appearance of an outstretched mantle with no signs of shrinking (1). The measurement of pH is a chemical analysis that can be used to measure fermentative-type of spoilage in oysters. It has been observed that the initial pH in fresh oysters is approximately 6.3 and a drop to approximately 6.0 - 5.8 is close to the limit of acceptability (24, 65). In most cases, sensory and chemical analyses are the consequence of microbial degradation; thereby the microbiological evaluation of stored oysters is often performed in order to have a better understanding of oyster shelf-life (23, 58).

#### 1.3.1 Oyster spoilage

Oyster spoilage is generally caused by the growth of micro-organisms to certain levels resulting in unpleasant sensory changes. Different sensory manifestations observed in microbiological spoilage of fish products include production of off-odours and -flavours, slime formation and discolouration (90).

The dominant group of bacteria found in fish under aerobic chilled storage are *Pseudomonas* and *Shewanella putrefaciens* (53). However, the types of bacteria associated

with spoilage of oysters might differ to marine fish, as oysters can survive out of water for several weeks (203). Moreover, the chemical composition of oysters includes a significant content on carbohydrates (up to 6.8%) mostly in the form of glycogen and a lower quantity of nitrogen in comparison to other seafood. Oyster spoilage is basically fermentative and production of acid is expected due to the breakdown of the glycogen which will enhance the proliferation of low pH tolerant bacteria (49).

### Bacterial composition

The microflora of oysters at harvest represents a combination of the micro-organisms that have been filtered from the water and the commensals microflora of the oyster. For example, *Cristispira* is an as yet unculturable spirochaete which forms part of the digestive system in oysters (136). The bacterial composition of shellfish related to the oyster habitat can vary depending on different factors such as salinity, environmental condition, bacterial load in the water, water temperature, diet, method of catch and chilling conditions.

Therefore, it is expected that bivalves from various locations or from the same location at different times may show a different bacterial concentration or composition.

The bacterial diversity in seawater and oysters has been compared in different studies. Although microbial flora of seawater and oysters was thought to be similar in early studies (221), differences in diversity and dominant species have been found. Specifically, bacterial diversity in seawater is found to be higher than in the oyster, while the numbers for a determinate group of bacteria are usually higher in the oyster in comparison to seawater. This indicates that only certain bacteria can survive and proliferate in the oyster.

Oysters provide higher organic level, lower level of oxygen and more constant salinity levels. Differences in diversity and numbers are also found among oyster tissues in which the oyster gut supports a more distinct bacterial flora than the rest of the animal (31, 120).

Microbial flora of PO is dominated by Gram negative bacteria including *Pseudomonas* and *Vibrio* species. Other reported bacteria isolated from oysters are *Achromobacter*, *Flavobacterium*, *Corynebacterium*, *Alcaligenes*, *Shewanella*, *Enterobacteriaceae*, *Moraxella*, *Staphylococcus*, *Micrococcus*, *Acinetobacter*, *Aeromonas*, *Bacillus* and lactic acid bacteria (42, 46, 120, 221).

After storage, the conditions in the oyster change which may enhance the proliferation of specific bacteria resulting in a reduction in diversity. Studies in PO have observed that *Lactobacillus* appear to be the major component after two days storage at 7°C (206) while a significant increase in *Pseudomonas* has been observed after storage at 5 and 10°C (42, 43).

#### Molecular methods to measure microbial communities

Different methods are available for microbial analysis and each one has limitations. Some bacteria are not easily cultured using standard culture-dependent methods due to the need for special agar composition or storage conditions, and in other cases there is selection in which growth of determinate bacteria is best suited to the nutrient, temperature and time given for a specific incubation. Thereby, one would expect to observe a less diverse

community where culture-dependent methods are employed in comparison to molecular methods.

A potential drawback of molecular-based methods using DNA extraction is detection of non-viable bacteria in the sample. In oysters, the majority of studies have been based on culturable bacteria. However, it is observed that some bacteria present in oysters can not be cultured in conventional agars (191, 192) and recently, limitations of general growth media have also been observed in seafood quality studies (37). Microscopic observations of total bacteria present in oyster have shown that there are  $10^5$  times more bacterial cells per gram than observed by plating (191).

The use of molecular techniques has provided a better understanding of the bacterial diversity in oysters. The bacterial composition of SRO was identified using 16S rRNA gene-based clone libraries and showed the bacterial community to be composed of 44% *Firmicutes*, 36% *Proteobacteria* (mostly of class *Alphaproteobacteria*), 7% *Cyanobacteria* and 5% *Spirochaetes* (92). *Arcobacter* spp. a potentially pathogenic member of class *Epsilonproteobacteria* were found to be abundant in depurated Chilean oysters (*Tiostrea chilensis*) (192). The use of molecular techniques has also been applied in oyster spoilage studies, identifying *Pseudoalteromonas* spp. as an abundant bacterial group in Chilean oysters stored at 18°C (193).

### Indicator organisms

Indicator organisms are typically used to reflect the microbiological quality or safety of a food product. The presence of an indicator organism at a certain level can provide an estimation of the quality or shelf-life of the food product. In general, the most reliable indicators tend to be product-specific (110).

An estimation of Total Viable bacteria Count (TVC) is used as an index in many seafood standards (107). Microbial criteria for satisfactory oysters at the wholesale level have been set at 5.7 - 6.2 log<sub>10</sub> CFU/g in the USA (15, 21). However, a higher level of 10<sup>7</sup> log<sub>10</sub> CFU/g TVC has been found to correlate with the maximum shelf-life for purified PO stored at cold temperatures (0 - 10°C) (43).

In some cases, only a fraction of the total flora induces the major changes and thereby counts of Specific Spoilage Organisms (SSO) are better related to shelf-life than TVC (26, 106). The SSO approach is also used in predictive models for shelf-life (54).

## **1.4 Supply chain management**

Supply chain management is the management of upstream and downstream relationships with suppliers and costumers to deliver high-value products at low cost for the supply chain. Ideally, the chain members should act as if the whole chain was only one single company, to optimize cost and share benefits (78).

Food safety management is an important aspect of the food supply chain. A lack of this discipline could result in a loss of public confidence and trust in the safety of food. An example of food crisis was where bovine spongiform encephalopathy caused by a fraudulent activity during primary production translated into a decrease of beef consumption by 11% throughout the European Union in 1996 (121).

### Cold supply chain

The growth of pathogenic and also spoilage micro-organisms can occur faster when those organisms are present in the product and the storage and transportation of foods take place under improper conditions. In the case of *V. parahaemolyticus*, it can multiply rapidly in foods if not refrigerated properly (84). Thus increases in temperature in supply chains can expose consumers to elevated risk and also adversely affect the product appearance and/or shelf-life. Proper supply chain management can help to maintain both quality and safety of oysters. However, maintaining proper temperature throughout a product shelf-life, in both storage and transit, can be complicated.

One of the issues in the cold supply chain is cost due to the necessary specialised transportation equipment and storage facilities which are different to that used in ambient supply chains, especially during summer. Apart from the cost of maintaining low temperature, there are situations in which temperature control is difficult (e.g. loading and unloading trucks, storage without a chill reception area, and transport to the home) (207).



### The oyster supply chain

Like other perishable products, oysters require a cold supply chain. However, the requirements are higher in the case of oysters because they are a live product. There is a lack of “use-by” date or “best before” date for oysters but they usually have a maximum of seven to eleven days from harvest to consumer depending on storage temperature (42, 43).

Manufacturers and retailers need to follow temperature guidelines. However, it is possible that temperature during storage, transport, retail display and at home can deviate from the recommended range. Market surveys in the USA shows that oyster lots exceeded the recommended 10°C reaching a maximum temperature of 16 - 17°C in some occasions (22, 50, 62). Similarly, oyster lots exceeding the recommended temperatures have also been observed in Australia during supply chain studies (132).

The quality and safety of the oysters will depend on the proper handling during all the different segments of the supply chain in which consumer plays a big role. Surveys of consumer refrigerators in Europe and Australia show that temperatures can sporadically reach 18 to 20°C (8, 117, 137). This is an important issue as if the cold chain is interrupted during transport to or at home, the efforts through all prior stages may be negated. Food companies spend money to ensure integrity of the product and they do not want to recall products that have been mishandled during the last part of the supply chain.

### The oyster supply chain in Australia

The oyster supply chain in Australia is complex due to the multiple transactions between the grower and consumer. It is also characterised by a large number of producers, many of whom are small and act independently. The Australian oyster supply chain can be broken into four segments: oyster producers, directly related marketing intermediaries (e.g. oyster feeding, water-quality), mid-chain (e.g. brokers, wholesalers, oyster openers, distributors) and end-users (e.g. store retailers, fishmongers, independent retailers, restaurants, fish and chip operators, pubs and clubs) (47).

#### **1.4.1 Traceability**

The primary purpose of traceability systems in the food supply is to recall defective or hazardous products and to identify the source of the problem. On the other hand, the implementation of proper traceability systems enhances cooperation among different steps of the supply chain, improving supply chain management and providing a better control of the product quality.

Traceability is defined by ISO 9000:2000 as the ability to trace the history, application or location of that which is under consideration. It can be related to the origin of materials forming the product, the process history and the location of the product. Traceability downstream is termed “trace” and is used when the history of product origin is investigated (i.e. to define the operations in which contamination happened). Traceability upstream is called “track” and is used for determining its history after delivery (i.e. to locate the

product which is contaminated). An effective traceability system in a food supply chain provides brand protection and integrity, increases efficiency of the supply chain, implements recall and meets statutory obligations for key markets (217).

### Identification systems for traceability

There are different elements in traceability: identification (e.g. barcodes, radio frequency identification device), administration (e.g. warehouse management system), communication (e.g. e-mail) and supporting infrastructure (e.g. wireless network). Some advantages and disadvantages of identification systems are (19, 217):

- Paper-based system does not need special equipment and can be applied to all types of products and processes. However, it is labour intensive, transcription errors may occur, records can be lost and it requires extensive filing space and allocation of personnel for record keeping.

- Bar codes can be read quickly and automatically throughout the supply chain. However, the use of bar codes can imply the loss of independence in choice of labelling format and it needs investment in specialised equipment.

- Radio Frequency Identification Device (RFID) tags consist of two parts: a microchip with memory and other electronics and an antenna that enables the electromagnetic coupling between the microchip and a reader device (2). An advantage of RFID is the capture of additional data (e.g. temperature) which can be retrieved with product out of line-of-sight, reducing the labour throughout the process. It allows the

product to be followed in real-time across the supply chain and tags can travel through different environments. An important consideration for the success of the application of this technology is standardisation. In this respect, EPC global promotes the standardisation of RFID-supported processes (152). However, the need for a large investment, the cost of the tags and the fact that they do not allow item-level identification can be an inconvenience for some industries (154).

### Traceability systems

There are different organizations which can help with traceability implementation. The global system GS1 is a worldwide accepted system of standards for accurate identification and communication of information on products, assets, services and locations. GS1 systems use bar codes, RFID and a website page which can be used to enter, validate, store and maintain all information in a single location (<http://www.gs1au.org/>, [accessed 20/09/11]). Other organisations which can help to implement traceability, providing software and other management tools, include WiseFish (<http://www.wisefish.com/>, [accessed 20/09/11]), Tracetracker (<http://www.tracetracker.com/>, [accessed 20/09/11]) and InformationLeader (<http://www.informationleader.com/>, [accessed 20/09/11]).

A specific example of a food-based traceability system is Smart-Trace<sup>TM</sup> which uses disposable wireless sensors (Smart-trace tags) and wireless network, radio or satellite communication to send identity, location, and temperature data of the product from the trip origin to the destination. This traceability system has been positively evaluated for its use with predictive microbiology for the distribution of meat in Australia (151). However,

Smart-Trace™ is still being under development (<http://www.smart-trace.com/mission.php>, [accessed 31/05/11]). Another example evaluated fish supply chains is Info-Fisk, an internet-based system that relies on bar codes and data loggers. The system locates the product and transmit temperature measurements or other information along the whole chain in real-time (79).

### “Freshness” systems

Traceability plays an important role in the food supply chain, and in the case of perishable products, freshness is also an important requirement. Freshness is in many cases dependent on storage time and temperature.

Time-Temperature Integrators (TTI) are devices, usually in form of labels adhered to the product, which can monitor thermal history. They are programmed according to kinetics of the change in a quality index allowing a chromatic variation proportional to time-temperature exposure. The advantage of this application is its low cost and the possibility of positioning locally on the product. There are many available TTI based on molecular diffusion (3M Monitor Mark™), polymerization reactions (Fresh-Check®), enzymatic activity (CheckPoint®) and microbial growth (TRACEO®) (215). Some of the disadvantages are the need to determine the shelf-life of each product to find a suitable quality index and the fact that they do not show in which part of the supply chain the colour has changed. The applicability of TTI to monitor temperature for bacterial spoilage estimation has been previously studied in chilled, fresh and atmosphere modified packaged fish (170, 214, 218).

### Traceability in the Australian oyster industry

Australia has requirements for traceability systems as part of the primary production and processing standard for seafood (FSANZ FSC 4.2.1) and for exports under the Export Control (Fish and Fish products) Orders (2005).

Currently, the traceability system normally used for identification in the oyster supply chain is paper-based that records information regarding growers name, lease number, harvest date and description of the product. As it is recognized that oysters from different leases may be mixed by many wholesalers, identifying a grower linked to inadequate product quality or safety is nearly impossible (47).

An analysis of the Australian oyster supply chain shows that it will be unlikely that mid-chain or end users will introduce full traceability back to the grower, and that growers would be the ones that will need to assess the cost-benefit of its enforcement, and may only happen if traceability mechanisms are enforced by government legislation (47). The producers need to be aware that although there is a cost to implement traceability, an economic benefit is guaranteed by reducing the quality controls, recalling only products affected, introducing provenance-brand selection and identifying which is the responsible for a decrease in quality or safety due to improper product handling (78).

## **1.5 Predictive microbiology**

Through the use of mathematical models, the viability of bacteria can be estimated to minimize risk, enhance product quality and manage supply chains (66). Predictive

microbiology involves the use of mathematical expressions to describe microbial growth responses to environmental factors. Predictive microbiology is based on the premise that the responses of populations of micro-organisms to environmental factors are reproducible and that, by characterising environments in terms of those factors that most affect microbial growth and survival, it is possible from past observations to predict the responses of those micro-organisms in other, similar environments (147).

Since the development of “modern” predictive microbiology in 1960-1970 (148), many predictive models have been developed in broth and food matrices and many “user-friendly” applications have been designed which are currently expanding as tools for a proactive risk management of microbial issues.

### Classification

Microbial predictive models can be classified using different approaches: growth or inactivation depending on microbial behaviour; probabilistic or kinetic depending on the mathematical approach; or as primary, secondary and tertiary based on the types of parameters described (39).

- The primary models describe the evolution in the amount of bacteria over time under a specific set of conditions. The modified Gompertz equation is commonly used to describe the sigmoid growth curve shape (144). Another growth model for predicting microbial growth was defined by Baranyi et al. (28). In this model, the growth rate is determined by the cell concentration, the extracellular environment and the

physiological state of the cells. Many researchers have used the Baranyi model in specific microbial modelling applications and found in comparison to the Gompertz function, and other models, that it gives better results. Another reason for the wide use of Baranyi as a primary model is the availability of the program DMFit which can be used to fit the Baranyi model easily to datasets (144).

- Secondary models describe the effect of intrinsic (e.g. pH,  $a_w$ ) and extrinsic (e.g. temperature, atmosphere composition) factors on the organism growth or survival characteristics. In most predictive microbiology models, temperature represents the major environmental parameter and NaCl the most important food component influencing the kinetics of micro-organisms (196). There are different secondary models which can be used to model growth and inactivation of bacteria. The square-root, the polynomial and the gamma are examples of secondary models used commonly to model growth rate, generation time and lag time (40, 68, 196). The use of probabilistic models is also convenient to predict the likelihood of a microbial event occurring. Some applications include modelling growth/no growth interface and time-to-toxin production (196).

- Tertiary models are typically software interfaces in which the primary and secondary models are integrated into a ready-to-use application tool. Computer software programs provide an interface between the mathematic terms and the user, allowing model inputs to be easily entered and estimates to be observed in simple graphical outputs (210). An example of specific programs are the Seafood Spoilage and Safety Predictor (SSSP, <http://sssp.dtuaqua.dk/>) which was developed to predict and illustrate the effect of constant and fluctuating temperatures on growth of SSO and on remaining shelf-life of different seafood products (56).



*V. parahaemolyticus* models

A comprehensive understanding of the response of *V. parahaemolyticus* to environmental temperature is the basis for developing effective risk management strategies. Some mathematical models for the prediction of *V. parahaemolyticus* viability as a function of temperature are available. However, there are few models for viability of *V. parahaemolyticus* in live oysters, and none for *V. parahaemolyticus* in live PO.

In bacteriological broth systems, the growth of *V. parahaemolyticus* over a temperature range of 8 - 45°C (155) and the growth rate and lag time from 10 - 30°C have been modelled (233) (Table 5). In food matrices, predictive models for *V. parahaemolyticus* in a Korean oyster slurry over a temperature range of 20 - 30°C (233) and in salmon meat from 0 - 35°C (231) have also been reported. The USA Food and Drug Administration (USFDA) provides a model for the growth of *V. parahaemolyticus* in American oysters (*Crassostrea virginica*) in the USNSSP (22). A software program for the prediction of *V. parahaemolyticus* growth in broth systems at different temperature in the range 15 - 30°C, different pH and salt concentration conditions has been developed recently by Fujikawa et al. (80).

**Table 5.** A summary of secondary models for *V. parahaemolyticus*

Study description			Secondary model
Reference	System	T range (°C)	
Miles et al. 1997 <sup>a</sup>	<i>Broth</i>	8 - 45	$r(\log/h) = [0.03563 \times ((T + 273.15) - 278.5) \times ((1 - \exp(0.3403 \times ((T + 273.15) - 319.6)))) \times \sqrt{((0.95 - 0.921) \times ((1 - \exp(263.64 \times (0.95 - 0.998))))}]^2 \times 60$
Yoon et al. 2008 <sup>p</sup>	<i>Broth</i>	10 - 30	$r(\log/h) = (0.00219 \times (T - 6.128))^2$
Yoon et al. 2008 <sup>p</sup>	<i>Korean oysters</i>	20 - 31	$r(\log/h) = (0.00723 \times (T - 20.31))^2$
Yang et al. 2009 <sup>p</sup>	<i>Salmon</i>	0 - 35	$r(\log/h) = (0.00421 \times (T - 12.057))^2 \times 2.302$
Anonymous 2007	<i>American oysters</i>	10 - 37.8	$r(\log/h) = (0.01122 \times ((T \times (9/5) + 32) - 0.4689))^2$

a: water activity value set to 0.95; p: pathogenic strain. Source data: various authors (22, 155, 231, 233).

### Evaluation of predictive models

Models built in relatively simple systems (e.g. broth) do not consider the effect of other micro-organisms, the food physical structure and levels of other chemical agents potentially present in the food product. For this reason, models can provide a better performance when developed for a specific intended food product (150).

In all cases, the accuracy of the model needs to be considered and it can not be used in real situations until it has been properly evaluated. This demonstrates the limitation of the model and if changes need to be done to increase its applicability. For food safety risk management, errors in the estimate of growth should tend toward a faster growth rate to provide a “fail-safe” prediction (229). However, a highly “fail-safe” model can also be too conservative that it becomes impractical for industry application. In fact, this situation

could cause unnecessary inspections or even recall of products from the market that may be perfectly safe. Commonly used measures to evaluate model performance objectively are the bias and accuracy factors (194).

### Applications

In general terms, predictive microbiology provides support for food quality and safety management strategies. Predictive models are very useful tools which have been proven to help different sectors: industry (143), regulatory authorities and science (150, 229). A list of specific applications is described in Table 6.

**Table 6.** Predictive microbiology applications

---

<b>HACCP</b>	Hazard Analysis Critical Control point: used as a tool for safety management Identification of the hazard that will grow and how fast Identification of steps in which grow is possible and establishment of critical control points Assessment of different scenarios Detection of the appropriate corrective measures
<b>RA</b>	Risk Assessment: used as a tool for safety management Estimation of changes in microbial numbers in different operations Assessment of exposure to a particular pathogen
<b>R&amp;D</b>	Research and Development: usually scientific field Prediction of the time until spoilers, pathogens or toxins reach dangerous level Study the effect of altering product composition or processing Evaluation of effect or out-of specification circumstances Optimal conditions to inhibit pathogen or spoilage organisms As a reference to design new experiments or new products
<b>EDU</b>	Education: usually for industry Show the influence of temperature on microbial growth by graphs to non-technical people Demonstrate the importance of maintaining proper refrigeration temperatures Observe consequences of changing formula composition in foods Recommendations in if-then questions Study different operations

<b>SC</b>	Supply Chain
	Integration into temperature loggers for pathogen growth control
	Integration with remote traceability for real-time monitoring of pathogen growth

---

Source data: various authors (143, 145, 146, 229).

There are some considerations for the application of predictive microbiology. Models need to be properly evaluated in the food product which usually involves more challenge studies to approve its applicability. Another consideration is that the person using the models needs to interpret correctly the output which includes knowledge of the model limitations (e.g. knowledge of the boundaries beyond which predictions should not be made) (150).

### 1.5.1 Risk assessment

Risk managers use risk assessments to manage risk to an acceptable level. Risk assessment is a scientifically-based process which consists of four different elements (27):

- Hazard identification in which the connection between disease and presence of a pathogen in food is documented
- Hazard characterisation that aims to estimate the nature, severity and duration of the adverse effects resulting from ingestion of the hazard
- Exposure assessment that tracks the pathways by which the pathogen enters the food supply and multiplies, survives or dies until the food is consumed in order to estimate the likely consumption of the pathogen

- Risk characterisation that integrates the information from the previous steps to estimate the risk in terms of the likelihood and severity of illness

Predictive microbiology can be used to relate the levels of a pathogen in a food depending on its conditions which is an essential part of the exposure assessment in microbial food safety risk assessments (195).

#### Deterministic and probabilistic risk assessment

Deterministic assessment is commonly used as a first step in exposure assessments because they are relatively quick, simple and inexpensive. It assumes that all individuals consume the specified food at the same level, that the hazard is always present in food, and that it is always present at a determinate concentration (27, 66). Deterministic models do not predict probabilities but changes in concentrations and they can not be directly implemented in Quantitative Microbial Risk Assessment (QMRA) (158).

Probabilistic assessment is used in QMRA to include variability and uncertainty.

Stochastic model distributions are used to generate the range of likelihood of possible outcomes from the overall processes analysed. In probabilistic modelling, the variables are described in terms of probability distributions instead of point estimates and the outcome is a risk distribution. An important advantage of probabilistic risk assessment is that it allows consideration of the whole distribution of exposure, from minimum to maximum. This probability is a quantitative measure, a number between zero and one expressing the odds

of an event. Thereby more meaningful information is provided to risk managers and the public. Some disadvantages are the need for accurate prediction of the tails in a distribution, the degree of complication and the time required to select and fit probability distributions (66).

Monte Carlo simulation, a computational method used to achieve multiple samples of the input distributions, is the method most commonly used for classical probabilistic risk assessment. It selects one random sample from each input distribution and the set of samples is entered into the deterministic model. The model is then solved, as it would be for any deterministic analysis and the result is stored. This iterative process is repeated several times until the specified number of iterations is completed. This method is described as a first order Monte Carlo simulation (27, 66). One of the disadvantages of using Monte Carlo simulations is the need of precise probability distributions for all inputs parameters (159). The process of setting up and running stochastic models require appropriate modelling software and a high level of computer processing power. There are a variety of risk analysis software products on the market, examples are @RISK, Crystal Ball, FARE Microbial<sup>TM</sup> and ModelRisk<sup>®</sup> (27, 66).

In QMRA, probability distributions are used to represent either variability or uncertainty. Variability represents inherent heterogeneity or diversity in a population (e.g. differences among strains, within strains, composition of the food) and uncertainty represents lack of information (e.g. measurement error, assumptions). Variability and uncertainty are easily confused because they are both represented by probability distributions and the difference between the two is not always obvious. Thereby they are not separated in most current QMRA (27).

However, lack of separation may lead to an incorrect interpretation of the results (161). It has been observed that the prediction of the outbreak size may depend on the way that uncertainty and variability are separated (160). To separate variability and uncertainty, a two-dimensional Monte Carlo simulation that consists in two Monte Carlo loops, one nested inside each other, is necessary. The inner loop deals with the variability of the input variables, while the outer one deals with uncertainty (27, 66).

An advantage of probabilistic risk analysis is the ability to perform sensitivity analysis to determine which variables in the model have the greatest influence on results. The results permit risk managers to consider different strategies for reducing exposure levels. Because the probabilistic analysis provides information on full distribution exposure, the exposure assessor can determine how different scenarios will affect different operations during distribution (27, 66).

#### *Vibrio parahaemolyticus* risk assessment

In order to have better management for *V. parahaemolyticus*, a Quantitative Risk Assessment on public health impact in raw oysters (VQRA) was developed in 2005 (17). The VQRA identified different factors that can markedly influence the presence and outgrowth of the bacterium in oysters, including water temperature, region and season during harvest, ambient air temperature after harvest as well as time between harvest and cooling. The VQRA is a scientific document that can be used for risk managers to establish regulations. At the moment, the document is mostly based on USA data and differences among countries and oyster species could be expected.

## 1.6 Objectives of this research

It has been estimated that 11,500 cases of food-borne disease occur every day in Australia, costing the community over 2.6 billion Australian dollars per year (9). The oyster industry is an important part of the economic seafood sector in Australia and it needs to supply the best quality and safest possible products to consumers.

Among the different microbial hazards, *V. parahaemolyticus* represents the leading bacterium involved in oyster-related outbreaks mainly due to raw or undercooked products.

An increase in the presence of *V. parahaemolyticus* can be expected due to the effects of climate change and it will probably lead to greater enforcement in order to protect public health. As such, the Australia oyster industry requires information about how to control *V. parahaemolyticus* growth in oyster species, as well as growth of TVC that influence product quality.

*V. parahaemolyticus* accumulation in oysters is practically unavoidable and pathogenic strains can reach infective levels with improper handling. There are different post-harvest treatments which can reduce the presence of this species but they can negatively affect the sensory properties or viability of oysters.

The aim of this project was to develop predictive microbiology tools to assist in supply chain management and thus improve the safety and quality of oysters in the marketplace.

Four different objectives were set in order to achieve the aim:



- Development and evaluation of a predictive model for the viability of *V. parahaemolyticus* and TVC in PO to predict their growth depending on storage temperature (Chapter 2).
- Evaluation of software for predicting *V. parahaemolyticus* and TVC growth in oyster supply chains as a function of temperature profile (Chapter 3).
- Production of a stochastic model to estimate the percentage of oysters containing specific bacterial levels in supply chain operations (Chapter 4).
- Measurement of the changes in bacterial communities in oysters under different storage temperatures (Chapter 5).

## **2 - Predictive models for the effect of storage temperature on viability of *Vibrio parahaemolyticus* and total viable bacteria count viability in the Pacific oyster (*Crassostrea gigas*)**

### **2.1 Introduction**

*Vibrio parahaemolyticus* is a bacterial species indigenous to marine environments and can accumulate in oysters (61). Some *V. parahaemolyticus* strains are pathogenic (232).

Consequently, the consumption of raw or improperly cooked oysters can result in *V. parahaemolyticus* infection (17, 23, 119, 130).

Disease occurs worldwide (209) with a higher incidence reported in Asia (208), South America (98) and the USA (58, 227). In Australia, *tdh+* *V. parahaemolyticus* has been isolated from oysters (125) and two reported outbreaks have been linked to oyster consumption, one death in 1992 and two cases in 2005 (6, 119). Therefore, the risk of *V. parahaemolyticus* infection in Australia is considered to be relatively low.

In response to *V. parahaemolyticus* risk associated with raw oyster consumption, the USFDA published a risk assessment in 2005 (17). The VQRA identified ambient air temperature after harvest as well as time between harvest and cooling as factors that can markedly influence the presence and outgrowth of the bacterium in oysters. Currently, the USNSSP and the ASQAP include guidelines for temperature during post harvest and distribution to control *V. parahaemolyticus* growth in oysters (4, 20).

However, a recent market survey of oyster microbiological quality performed in the USA found that 15% of tested lots exceeded the 10,000 MPN/g *V. parahaemolyticus* criterion recommended by the USFDA (22, 62). In addition, recent data from the CDC indicate that humans infections caused by *Vibrio* spp. have increased in relation to other food borne pathogens (16). Another important factor with the potential to affect *V. parahaemolyticus* exposure levels and alter geographical distribution is the influence of climate change (138, 140). This emphasises the need to improve risk management practices for this bacterium.

A comprehensive understanding of the response of *V. parahaemolyticus* to environmental temperature is the basis for developing effective risk management strategies for regulatory agencies, oyster producers and consumers. In this regard, predictive microbiology offers a systematic approach to describe microbial responses to different environments (147).

Through the use of mathematical models, the viability of pathogenic bacteria can be estimated to minimize risk, enhance product quality and manage supply chains (66).

Mathematical models have been developed for the prediction of *V. parahaemolyticus* growth as a function of temperature in bacteriological broth systems (155, 233) and in food matrices (231, 233). A mathematical equation for estimating the growth of *V. parahaemolyticus* in American oysters depending on temperature is also provided by the USNSSP (22). However, there are few predictive models for viability in live oysters, and none for *V. parahaemolyticus* in live PO.

Thus, the objective of this study was to develop mathematical models to describe the effect of storage temperature on the viability of *V. parahaemolyticus*. In parallel, a model was developed for TVC in live PO. The predictive microbiology models were evaluated in PO

and SRO containing indigenous *V. parahaemolyticus* harvested from a different geographical location.

## 2.2 Materials and Methods

**Oyster samples.** In experiments to produce the predictive model, ten batches of live PO were harvested by a commercial grower in Pipeclay Lagoon, Tasmania between September 2008 and December 2008. Following collection, oysters were placed in a cooler with gel packs and transported within 2 h to the laboratory at the University of Tasmania, Hobart, Tasmania. Oysters were washed with tap water to remove excess mud on the shells, as indicated by the American Public Health Association for the bacteriological examination of shellfish (105), stored at approximately 7°C and processed within 24 h of harvest. Seawater pH (Waterproof pHTestr1, Oakton, Vernon Hills, IL, USA), temperature and salinity (microprocessor conductivity meter model LF-196, WTW, Germany) and dissolved oxygen (microprocessor oximeter model OXI-196, WTW, Germany) were measured in the top 5 cm of the water surface in the harvest area at the time of sample collection. A total of 1600 oysters were used in experiments.

The model for *V. parahaemolyticus* viability in PO was evaluated in PO and SRO harvested in Port Stephens, New South Wales. Following harvest, oysters were packed in a cooler and the temperature monitored during transport using a temperature data logger (iButton<sup>®</sup>, Maxim Integrated Products, Inc., Sunnyvale, CA). Three different batches of SRO were harvested in April 2009, May 2009 and February 2010; one batch of PO was

harvested in February 2010. All batches were shipped to the laboratory in Hobart, Tasmania by overnight courier and tested within 26 h of collection. A separate batch of PO harvested in the same area in May 2009 was shipped by overnight courier to the South Australian Research and Development Institute (SARDI) Food Safety laboratory in Glenside, South Australia and tested within 24 h of harvest. A total of 1000 oysters were tested for all validation studies using New South Wales oysters.

**Bacterial strains.** Local strains with the desired combinations of *tdh* and *trh* genes were not available. Six *V. parahaemolyticus* strains (24339, 24340, 24657, 24658, 24659, 24660) were used to produce the PO model. The strains were isolated from shrimp in Thailand and kindly provided by Dr Orasa Suthienkul, Faculty of Public Health, Department of Microbiology, Mahidol University, Bangkok. For the purpose of these studies, the strains were coded as follows: 39 (24339), 40 (24340), 57 (24657), 58 (24658), 59 (24659) and 60 (24660). Cultures were stored at -80°C in modified Tryptone Soy Broth (mTSB; TSB [CM0129, Oxoid, Adelaide, Australia] supplemented with 3%NaCl and adjusted to pH 8.4) with addition of 15% (v/v) glycerol (Sigma-Aldrich, Steinheim, Germany).

**Colony PCR assay for species and virulence genes.** A multiplex PCR assay was performed to detect *tdh*, *trh* and *tlh* genes (167, 168, 213) in the six different bacterial strains used in the cocktail. Bacterial strains were cultured on TCBS agar (CM0333, Oxoid, Adelaide, Australia) to confirm that colonial morphology was typical of *V. parahaemolyticus* (115). For each assay, one isolated colony was mixed in 200 µl of

sterile distilled water, stored at -20°C and the sample suspension was used as template for the PCR assay without DNA extraction. The presence of the three genes was tested using a 20 µl reaction mixture containing 2 µl DNA template, 10 µl ImmoMix™ Red (Bioline, NSW, Australia), 0.25 µM of each oligonucleotide reverse and forward primer, and 5 µl of RNase-free water (BIO38031, Bioline, NSW, Australia). The oligonucleotide primers sequence used for *tlh* (F-*tlh*:5'ACTCAACACAAGAAGAGATCGACAA-3' and R-*tlh*:5'GATGAGCGGTTGATGTCCAA-3') were as reported by Nordstrom et al. (169), and the sequences for *tdh* (F-*tdh*:5'GTAAAGGTCTCTGACTTTTGGAC-3' and R-*tdh*:5'TGGAATAGAACCTTCATCTTCACC-3') and *trh* (F-*trh*:5'TTGGCTTCGATATTTTCAGTATCT-3' and R-*trh*:5'CATAACAAACATATGCCCATTTCCG-3') were as reported by Bej et al. (30). The three primers were commercially synthesized (Gene Works, SA, Australia).

The PCR cycle program consisted of a denaturation step at 95°C for 10 min followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final elongation at 72°C for 7 min. Strain 60 (*tlh*+/*tdh*+/*trh*+) was used as a positive control and RNase-free water was used as negative control.

Electrophoresis of PCR-amplified DNA (5 µl) was conducted at room temperature on a 2% (w/v) agarose gel containing GelRed™ Nucleic Acid Gel Stain (Biotium, California, USA) in 1x Tris-Acetate-EDTA (TAE) buffer at a constant voltage of 74 V for 80 min.

Visualization of DNA was performed with a transilluminator (Bio-Rad, NSW, Australia) and the image processed using Quantity One® 4.6.6 1-D Analysis software (Bio-Rad, NSW, Australia). PCR products for *tlh*, *tdh* and *trh* approximate sizes were approximately 200 bp,

250 bp and 500 bp, respectively when compared to the marker (HLII; BIO33039, Bioline, NSW, Australia).

**Preparation of *V. parahaemolyticus* injection inoculum.** For each experiment, each *V. parahaemolyticus* strain was transferred from -80°C storage to a plate of modified Tryptone Soy Agar (mTSA; mTSB with addition of 1.5% agar [grade J3, Gelita, QLD, Australia]) and incubated at 25°C for 18 - 24 h. For each of the six strains, two to three colonies from each of the mTSA plates were selected and enriched in 9 ml Marine Broth (MB). MB consisted of bacteriological peptone 0.5% (LP0037, Oxoid, SA, Australia), yeast extract 0.1% (LP0021, Oxoid, Adelaide, Australia), and sea salts 3.5% (w/v) (RedSeaFish, NSW, Australia) with pH adjusted to 8.4. The six broths were incubated at 25°C for 18 - 24 h. Each culture was adjusted to 0.15 - 0.25 OD<sub>540</sub> nm using 200 µl in a Benchmark Microplate reader (Bio-Rad, NSW, Australia). Two millilitre aliquots of each working culture were combined to produce a 12 ml cocktail of approximately  $3 \times 10^8$  total CFU/ml. The cocktail for oyster injection was diluted in sterile artificial sea water (3.5% sea salts w/v) to a final concentration of approximately  $1.5 \times 10^6$  CFU/ml. For low temperature storage studies (3.6, 6.2, 9.6 and 12.6°C), the inoculum was prepared to a concentration of  $1.5 \times 10^8$  CFU/ml to facilitate modelling inactivation. Inocula were kept on ice in tubes during the injection process for approximately 30 min. It is possible that a slight decrease in cells occurred due to low temperature sensitivity of *V. parahaemolyticus* (41), however the final required initial concentration in oysters was achieved.

**Oyster inoculation.** For each storage assay, an approximate 5 mm notch was drilled in the oyster shell approximately 50 mm from the hinge, based on average size of 80 mm oyster length, using a power drill (Dremel® Multipro 395, WI, USA), avoiding contact with oyster tissue (116). The adductor muscle of 60 oysters was then directly injected with 0.1 ml of the inoculum cocktail using a 1 ml syringe equipped with a 23-gauge needle (Terumo, USA), similar to a method previously reported (83). In a preliminary experiment, injection in the adductor muscle was compared to that in the visceral mass (Appendix A1). The mean of the ratio between counts for visceral mass and counts for adductor muscle were nearly identical at  $1.0 \pm 0.16$  for storage at 20°C and  $1.0 \pm 0.03$  for storage at 25°C. However, injection in the adductor muscle produced more consistent *V. parahaemolyticus* growth curves at the two different temperatures tested and therefore this tissue was used for inoculation. The remaining 100 oysters were injected with 0.1 ml of sterile artificial seawater.

**Storage conditions.** Injected oysters were stacked in 2-3 layers in open plastic containers and stored in incubators at  $3.6 \pm 0.1$ ,  $6.2 \pm 0.1$ ,  $9.3 \pm 0.3$ ,  $9.6 \pm 0.3$ ,  $12.6 \pm 0.4$ ,  $14.9 \pm 0.1$ ,  $18.4 \pm 0.2$ ,  $20.0 \pm 0.1$ ,  $25.7 \pm 0.2$ , and  $30.4 \pm 0.3$ °C. Storage time varied from 437 h at 3.6°C to 58 h at 30.4°C based on oyster viability during the course of experiments. When oyster sample shells gaped, oysters were not considered viable and experiments were not continued. Incubator temperature was monitored by placing temperature data loggers between oysters. The resolution of temperature loggers was 0.5°C. The mean  $\pm$  Standard Deviation (SD) for each storage temperature measured from the two different data loggers for each experiment was recorded.



In model validation studies, SRO oysters containing natural populations of

*V. parahaemolyticus* were stored at  $15.3 \pm 0.2$ ,  $18.0 \pm 0.1$ ,  $21.8 \pm 0.4$ ,  $24.2 \pm 0.3$  and  $27.9 \pm 0.2^\circ\text{C}$ , and PO at  $15.0 \pm 1$ ,  $18.0 \pm 0.1$ ,  $23.0 \pm 1$ ,  $24.2 \pm 0.3$  and  $28.0 \pm 1^\circ\text{C}$ . Experiments at 15, 23 and  $28^\circ\text{C}$  for PO were performed at SARDI laboratories.

**Bacterial enumeration.** Preliminary studies were conducted to determine the appropriate sample size of injected oysters for bacterial enumeration. Six separate samples of three oysters were tested immediately after injection and after storage at  $20^\circ\text{C}$  for 2 d. A SD of 0.12 at initial counts and a SD of 0.71 after oyster storage were observed among the six replicates, indicating a good repeatability of the method.

In model development studies, two separate samples of three oysters injected with *V. parahaemolyticus*, and one sample of ten seawater-injected oysters were analysed at selected time intervals. The larger number of controls was used to detect potential variability in background levels of indigenous *V. parahaemolyticus* (113). At the time of bacteriological analysis, oysters were opened aseptically with a sterile shucking knife as described by the American Public Health Association (105), meat and liquor placed in a sterile 400 ml filter stomacher bag (A.I. Scientific, Hallam, Australia) with an equal weight of Peptone Salt Solution (PSS; 0.1% bacteriological peptone [LP0037, Oxoid, Adelaide, Australia], 3% NaCl [Ajax Finechem, NSW, Australia], pH 7.4) and the sample stomached (Colworth Stomacher 400, A. J. Seward, London) for 2 min. Although buffered saline solution can be used as a diluent in *Vibrio* spp. assays (34, 115), the use of PSS is recommended in the Australian standard methods (AS/NZS 1766.2.9:1997). Due to the

high number of test samples per time interval, oysters were processed with a stomacher instead of a blender, as reported for other oyster studies (135). Stomached samples were diluted in 10-fold serial increments with PSS and 100 µl plated in duplicate on TCBS and on Marine Agar (MA; MB with addition of 1.5% agar [grade J3, Gelita, QLD, Australia]). TCBS and MA plates were incubated at 37°C for 16 - 18 h and 25°C for 48 h, respectively. Plated dilutions yielding 30 - 300 CFU/plate were counted manually and CFU/g of homogenate calculated.

In studies of oysters containing natural *V. parahaemolyticus* populations, *V. parahaemolyticus* was enumerated using a three-tube MPN method (115) with slight modification. Samples were homogenized and diluted as explained for seawater-injected oysters and the salt content of APW increased from 1% to 3% NaCl as this has been reported that improve isolation of *V. parahaemolyticus* (45). A 20-g sample of oyster homogenate was mixed with 80 g of modified APW (mAPW; 1% bacteriological peptone [LP0037, Oxoid, Adelaide, Australia], 3% NaCl [Ajax Finechem, NSW, Australia], pH 8.4) and then serial 10-fold dilutions prepared (v/v) in PSS. Ten and one millilitre from the initial dilution and one millilitre of all other dilutions were individually added to three tubes containing 10 ml mAPW. Inoculated mAPW tubes were incubated at 37°C for 16 - 18 h. A 100 µl sample of each turbid broth was transferred to a sterile non-skirted PCR 96-well plate (Bioline, NSW, Australia) and stored at -20°C for a maximum of one week until assayed by PCR. According to the presence-absence of positive bands in the agarose gel, total populations of *V. parahaemolyticus* were determined using a MPN table (115).

**MPN-PCR detection of *tlh*.** The presence of *V. parahaemolyticus* was carried out as described above for colony PCR but with some adjustments. Specifically, the primers directed to *tdh* and *trh* genes were omitted and the volumes in the mixture were changed as follows: 20 µl reaction mixture containing 2 µl sample, 10 µl ImmoMix™ Red, 0.1µM of each oligonucleotide reverse and forward primer, and 7.6 µl of RNase-free water. PCR cycles were reduced from 35 to 30 and electrophoresis of PCR amplified product applied for 30 min instead of 80 min. These conditions were adequate to separate bands and reduced assay time.

**Sensitivity of MPN-PCR for *tlh* detection.** The *V. parahaemolyticus* cocktail was prepared as for oyster-injection model studies and was 10-fold serially diluted in mAPW. One ml of each dilution was added to individual tubes containing 9 ml mAPW and 1.0 ml of a 10-fold dilution of oyster homogenate (0.1 g oyster/tube), giving final *V. parahaemolyticus* concentrations of 0 (negative control) to  $1.3 \times 10^6$  CFU/ml. Initially and after 16 h at 37°C, samples from each tube were divided into two groups; one group was boiled for 10 min at 90°C while the other was not boiled. Each sample treatment was tested by PCR detection for the *tlh* gene.

#### **Predictive models for *V. parahaemolyticus* and total viable bacteria counts.**

Plate count data were transformed to log<sub>10</sub> values. For growth profiles, data were fitted with DMFit curve-fitting software v2.1 (courtesy of the Institute of Food Research, Norwich) to estimate growth rate (log<sub>10</sub> CFU/h) using the growth model reported by

Baranyi and Roberts (28). For inactivation profiles, inactivation rates ( $-\log_{10}$  CFU/h) were estimated by linear regression using Microsoft Excel<sup>®</sup>.

For *V. parahaemolyticus* growth, the square root model (186) was used to describe growth rate ( $r$ ) as a function of temperature. The equation for the square root model is:

$$\sqrt{r} = b \times (T - T_0) \quad (1)$$

and shows a linear relationship between the square root of  $r$  and temperature ( $T$ ), where  $b$  is a regression coefficient and  $T_0$  is a hypothetical reference temperature which is an intrinsic property of the organism.

The Arrhenius equation (25) was used to estimate the kinetic parameters for the effect of temperature on bacterial inactivation. The equation used was:

$$\ln r = \ln A - E_a/RT \quad (2)$$

where  $r$  is the rate constant,  $T$  the absolute temperature,  $E_a$  the activation energy,  $R$  the universal gas constant and  $A$  the collision factor. The values of  $\ln k$  was plotted against  $1/T$  to calculate the values of  $E_a/R$  and  $A$  by linear regression using Microsoft Excel<sup>®</sup>.

The growth rate of TVC was plotted as a function of temperature and data fitted to the square root model as described for *V. parahaemolyticus*. All TVC and *V. parahaemolyticus* kinetic data have been submitted to ComBase, a database for predictive microbiology (29).

**Model performance.** Measurement of goodness-of-fit for each of the secondary models was done by evaluating the root mean square error (RMSE) and the coefficient of determination ( $R^2$ ) (185). Statistic information of the performance of the secondary models was obtained using regression data analysis in Excel<sup>®</sup> (Table A3.1). Evaluation of

secondary growth models for *V. parahaemolyticus* and TVC was performed with two different oyster species, PO and SRO, containing natural *V. parahaemolyticus* populations, during different seasons and from a different geographical location to that used for model development. The secondary model for *V. parahaemolyticus* growth was compared to other published *V. parahaemolyticus* models (22, 155, 231, 233).

## 2.3 Results

**Harvest conditions during oyster collection.** The average SST of the harvest area in Tasmania was  $15.8 \pm 2.2^{\circ}\text{C}$ , with an observed range of 12.3 to  $19.4^{\circ}\text{C}$ . The ranges of salinity and dissolved oxygen in the same area were 3.4 to 4.8‰ and 10.7 to 12.2 mg/l, respectively. The average pH was  $8.5 \pm 0.1$  (Table 7).

**Table 7.** Harvest conditions during oyster collection (oyster batches used during model development)

Collection date	Water measurements				Oyster size		Storage test
	T ( $^{\circ}\text{C}$ )	mg/l $\text{O}_2$	pH	Salt (%)	High	Wide	T ( $^{\circ}\text{C}$ )
26th November 2008	19.4	11.6	8.5	3.6	6.8	4.3	$3.6 \pm 0.1$
8th December 2008	15.0	11.5	8.6	-	7.6	4.9	$6.2 \pm 0.1$
29th September 2008	15.9	10.7	8.2	3.5	8.0	5.0	$9.3 \pm 0.3$
1st December 2008	13.8	-	8.5	3.5	7.8	4.5	$9.6 \pm 0.3$
3rd December 2008	13.6	12.2	8.5	3.6	8.2	5.4	$12.6 \pm 0.4$
20th October 2008	18.1	11.4	8.4	3.5	8.0	4.8	$14.9 \pm 0.1$
24th November 2008	16.9	11.0	8.5	3.5	7.4	4.8	$18.4 \pm 0.2$
13th October 2008	16.4	11.0	8.5	3.4	8.6	5.2	$20.0 \pm 0.1$
6th October 2008	12.3	12.0	8.5	4.8	8.6	5.6	$25.7 \pm 0.2$
1st October 2008	16.5	11.3	8.3	3.4	9.0	5.0	$30.4 \pm 0.3$

For the New South Wales harvest areas, SST averaged  $21.3 \pm 3.6^{\circ}\text{C}$ , with a range of 17.5 to  $25.5^{\circ}\text{C}$  and salinity  $3.1 \pm 0.6\%$  with a range from 2.4 to 3.7%; pH and dissolved oxygen data were not available (Table 8).

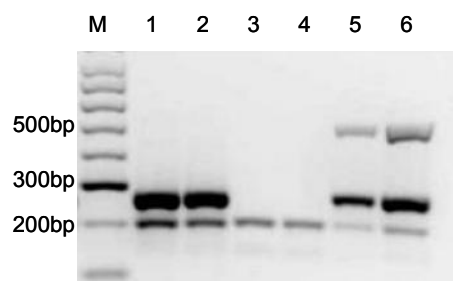
**Table 8.** Harvest conditions during oyster collection (oyster batches used during model evaluation)

Collection date	Transport Mean T $\pm$ SD ( $^{\circ}\text{C}$ )	Water T ( $^{\circ}\text{C}$ )	Salt (%)	Oyster size		Storage test T ( $^{\circ}\text{C}$ )	Oyster species
				High	Wide		
8th April 2009	$18.7 \pm 4$	21.0	3.0	8.8	5.2	$21.8 \pm 0.4$	SRO
13th May 2009	$13.7 \pm 2.9$	18.2	2.4	7.5	4.4	$27.9 \pm 0.2$ , $15.3 \pm 0.2$	SRO
24th May 2009	-	17.5	2.6	-	-	$15 \pm 1$ , $23 \pm 1$ , $28 \pm 1$	PO
15th February 2010	$25 \pm 2.8$	25.5	3.7	10	5	$18.0 \pm 0.1$ , $24.2$ $\pm 0.3$	SRO
22nd February 2010	$21 \pm 3$	24.5	3.7	9	5	$18.0 \pm 0.1$ , $24.2$ $\pm 0.3$	PO

**PCR assay for species and virulence genes, and sensitivity of MPN-PCR for detection of *V. parahaemolyticus* in injected oysters.** Multiplex-PCR tests confirmed that all strains possessed *tlh* (Figure 2). Strains 39, 40, 59, 60 contained *tdh*; strains 59, 60 *trh*; and strains 57, 58 were *tdh*- and *trh*-.

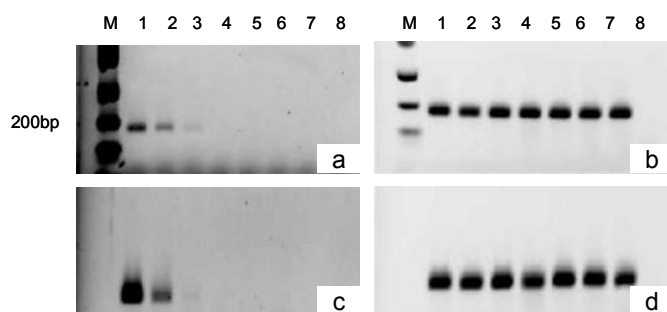
For the MPN-PCR method, results showed that the limit of reliable detection at the time of sample inoculation ( $t = 0$  h) was  $1.3 \times 10^5$  CFU/ml, regardless if samples were boiled (Figure 3a) or not boiled (Figure 3c). After approximate 16 h incubation, all boiled (Figure 3b) and non-boiled (Figure 3d) samples from the MPN tubes inoculated with 1.3 to

$1.3 \times 10^6$  CFU/ml the previous day, produced a positive PCR product. No band was observed for the negative control. The average level of *V. parahaemolyticus* in the inoculated overnight MPN tubes of mAPW incubated at 37°C for 16 - 18 h, as observed on TCBS plates, was  $8.6 \log_{10}$  CFU/ml.



Lane M: 2 kb molecular size marker; lane 1, strain 39; lane 2, strain 40; lane 3, strain 57; lane 4, strain 58; lane 5, strain 59 and lane 6, strain 60.

**Figure 2.** Agarose (2%) gel electrophoresis of *V. parahaemolyticus* PCR products corresponding to *tlh* (~200bp), *tdh* (~250bp) and *trh* (~500bp) genes.



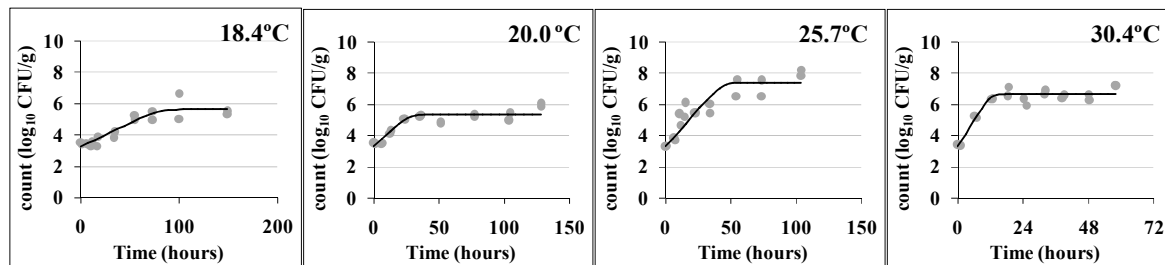
The oyster enrichments were tested after 0 h: with (a) and without (c) boiling; and after 16 h: with (b) and without (d) boiling. Lane M, 2 kb molecular size markers only used in the upper part of the agarose gel. Lanes 1-8, PCR products of oyster homogenate enrichment with contamination levels of *V. parahaemolyticus* cocktail of  $1.3 \times 10^6$ ,  $1.3 \times 10^5$ ,  $1.3 \times 10^4$ ,  $1.3 \times 10^3$ , 130, 13, 1.3 and 0 CFU/ml, respectively.

**Figure 3.** Agarose (2%) gel electrophoresis of *V. parahaemolyticus* PCR products corresponding to *tlh* gene (~200bp) in oyster enrichment samples inoculated with serial 10-fold dilutions of the *V. parahaemolyticus* cocktail.

**Primary models of *V. parahaemolyticus*.** For growth studies, oysters were injected with an average of  $3.4 \pm 0.1 \log_{10}$  CFU/g of the *V. parahaemolyticus* cocktail.

*V. parahaemolyticus* levels did not significantly increase or decrease at 14.9°C through 169 h. Therefore, growth rate was assumed to be 0  $\log_{10}$  CFU/h at this temperature. However, oysters stored at temperatures  $\geq 18.4^\circ\text{C}$  supported growth (Figure 4). The average coefficient of determination ( $R^2$ ) for the Baranyi model fitted to the four kinetic growth profiles was 0.86. Growth rates increased with increasing temperature, specifically 0.030, 0.075, 0.095, and 0.282  $\log_{10}$  CFU/h at 18.4, 20.0, 25.7, and 30.4°C, respectively.

Maximum Population Density (MPD) depended on storage temperature, with the highest levels (i.e. 7.4  $\log_{10}$  CFU/g) observed at 25.7°C after 73 h. A lag phase was not observed at any storage temperature.



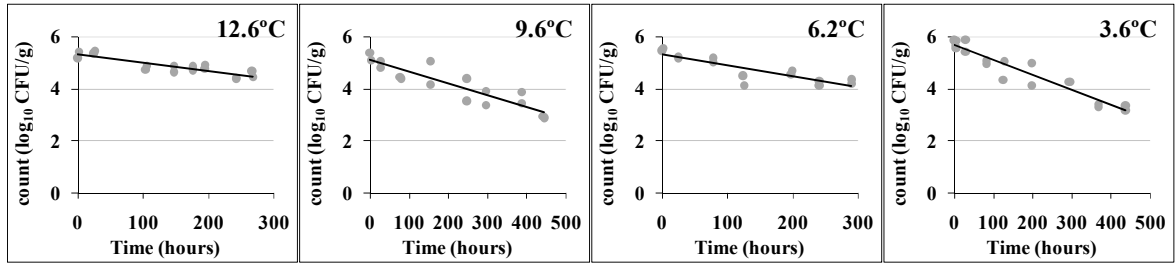
Count (●) in  $\log_{10}$  CFU/g and fitted curve (—).

**Figure 4.** Growth of *V. parahaemolyticus* in live Pacific oysters (18.4 - 30.4°C).

At temperatures resulting in *V. parahaemolyticus* inactivation (i.e. 3.6 to 12.6°C), oysters were injected with an average  $5.4 \pm 0.2 \log_{10}$  CFU/g. A linear regression fitted to the data showed an average  $R^2$  value of 0.82 (Figure 5). Inactivation rate values were -0.006, -



0.004, -0.005 and -0.003 log<sub>10</sub> CFU/h at 3.6, 6.2, 9.6, and 12.6°C, respectively. The highest, although relatively low, inactivation rate was observed at 3.6°C, with an approximate reduction of 2.5 log<sub>10</sub> CFU/g after 437 h. Inactivation below the detection limit (600 CFU/g) was not observed at any storage temperature for the duration of the experiment.



Count (●) in log<sub>10</sub> CFU/g and linear regression fit (—).

**Figure 5.** Inactivation of *V. parahaemolyticus* in live Pacific oysters (3.6 - 12.6°C).

#### Secondary models for *V. parahaemolyticus* viability in Pacific oysters.

Secondary models were produced for both *V. parahaemolyticus* growth and inactivation.

For growth, the square root of the growth rate was plotted as a function of temperature.

The square root model was fitted as shown in Figure 6a. The estimated values for

parameters  $b$  and  $T_0$  were 0.0303 and 13.37, respectively (Equation 3). Goodness-of-fit

comparing observed and predicted values showed a RMSE of 0.05 and a  $R^2$  value of 0.92.

$$\sqrt{r} = 0.0303 \times (T - 13.37) \quad (3)$$

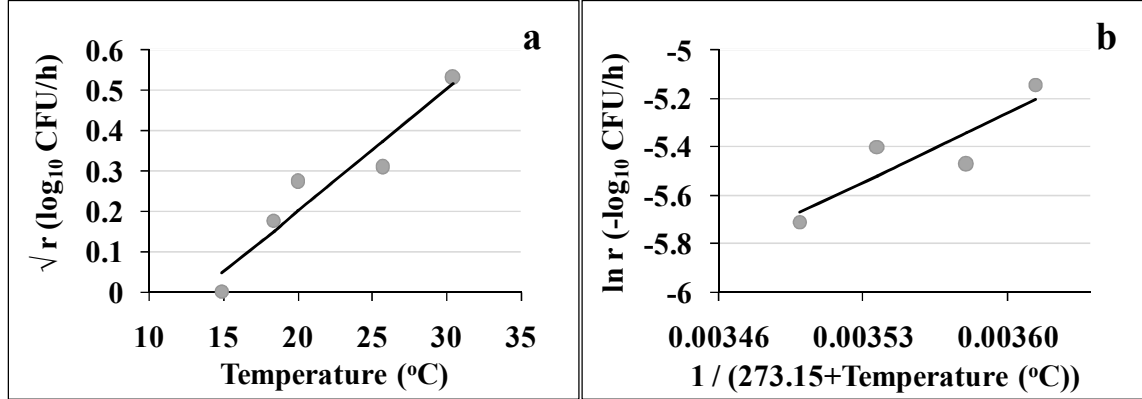
A linear Arrhenius model was used to describe the change in *V. parahaemolyticus*

numbers ( $\ln(-\log \text{CFU/h})$ ) from 3.6 and 12.6°C as a function of temperature ( $1/$

$(T+273.15)$ ), as shown in Figure 6b. Estimated values for terms  $E_a/R$  and  $A$  were 4131.2

and  $1.81 \times 10^{-9}$  respectively (Equation 4). Analysis of predicted and observed values was performed. The RMSE of the fitted model was 0.09 and the  $R^2$  was 0.78.

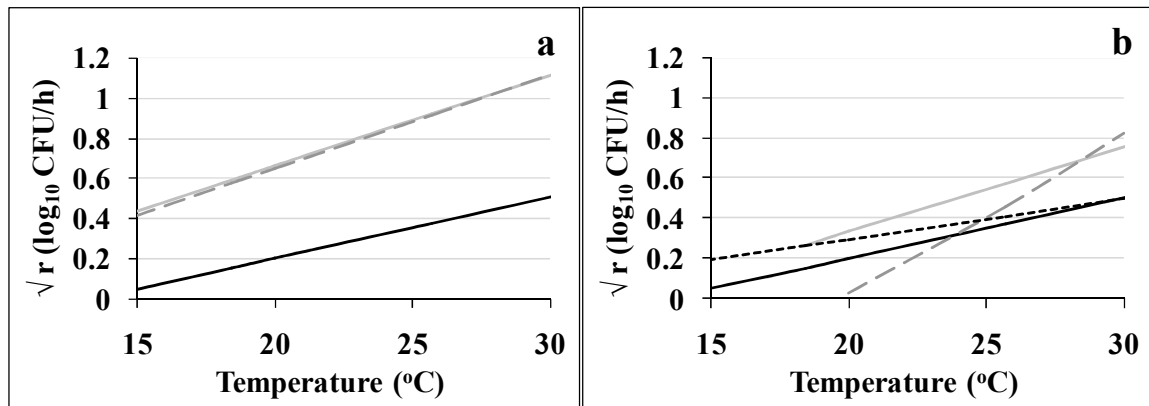
$$\ln r = \ln 1.81 \times 10^{-9} + 4131.2 \times (1/(T+273.15)) \quad (4)$$



Observed growth or inactivation rates (•) and model fit (—).

**Figure 6.** Secondary models for *V. parahaemolyticus*: growth (a) and inactivation (b).

The square root model (Equation 3) was compared to other published *V. parahaemolyticus* models (22, 155, 231, 233). Growth of *V. parahaemolyticus* in live PO was slower than that reported for bacteriological broth, salmon meat and for American oysters (*Crassostrea virginica*) between 15 and 29°C. In comparison to oyster slurries, the model deviated between 20 and 24°C, showing faster growth in live PO (Figure 7).



Models for *V. parahaemolyticus*: (a) (Equation 3) (—); broth system reported by Yoon et al. (233) for pathogenic strains (---) and by Miles et al. (155) (....). (b): (Equation 3) (—); model for a Korean oyster slurry reported by Yoon et al. (233) for pathogenic strains (---), model in salmon meat by Yang et al. (231) (....) and the model reported in the USNSSP (22) for American oysters (...).

**Figure 7.** Comparison of different secondary models for *V. parahaemolyticus* growth.

### Evaluation of the secondary *V. parahaemolyticus* growth model in Pacific and Sydney

#### Rock oysters containing indigenous *V. parahaemolyticus* populations. The growth rates

of natural populations of *V. parahaemolyticus* were tested at five different storage

temperatures for PO and SRO. *V. parahaemolyticus* multiplied in PO at 23 and 28°C, at

0.034 and 0.198 log<sub>10</sub> MPN/h, respectively. These growth rates were slower than model

predictions. Populations increased from 2.4 log<sub>10</sub> MPN/g to 4.4 log<sub>10</sub> MPN/g over 60 h

storage at 23°C, and to 4 log<sub>10</sub> MPN/g after 40 h storage at 28°C. In contrast, no significant

increase in *V. parahaemolyticus* levels was observed in PO stored at 15, 18 and 24.2°C.

For these temperatures, average *V. parahaemolyticus* densities were 2.9 ± 0.4, 3.3 ± 0.3

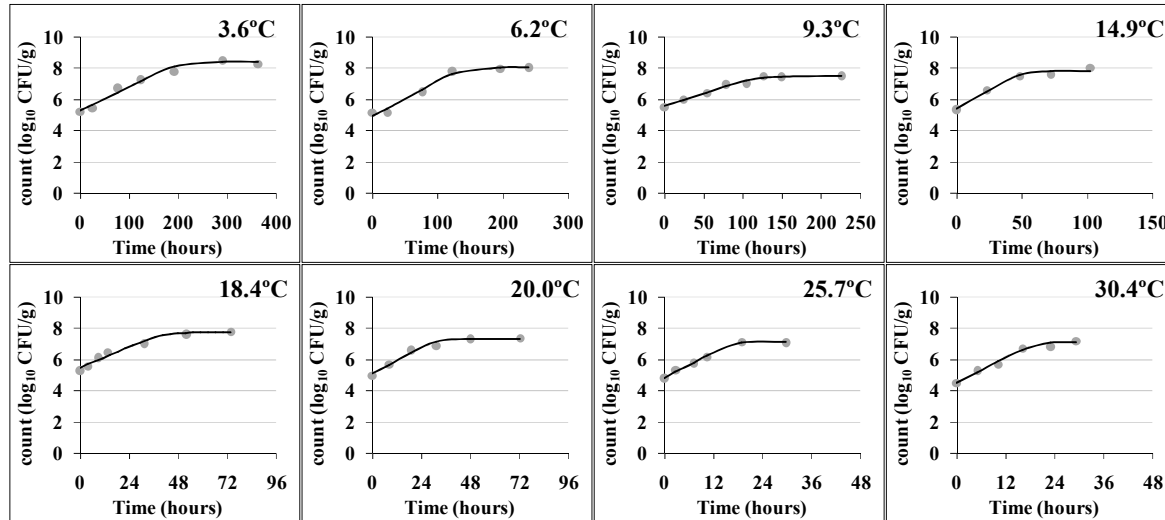
and 3.3 ± 0.4 log<sub>10</sub> MPN/g, respectively, over the duration of storage.

Opposed to PO, *V. parahaemolyticus* did not grow in SRO at any tested storage

temperature. The average *V. parahaemolyticus* levels during storage were 2.6 ± 0.4 at

15.3°C,  $2.4 \pm 0.5$  at 18°C,  $3.8 \pm 0.3$  at 21.8°C,  $2.6 \pm 0.7$  at 24.2°C and  $3.0 \pm 0.4$  log<sub>10</sub> MPN/g at 27.9°C.

**Primary models for total viable bacteria count.** The kinetics of TVC growth were determined using the seawater-injected control oysters, performed in parallel to measuring *V. parahaemolyticus* in injected oysters. Growth on MA was observed at all storage temperatures tested (3.6 - 30.4°C). The average R<sup>2</sup> value for the Baranyi model fitted to the eight kinetic growth profiles was 0.97 (Figure 8). Growth rates were 0.015, 0.023, 0.016, 0.048, 0.055, 0.071, 0.139 and 0.135 log<sub>10</sub> CFU/h at 3.6, 6.2, 9.3, 14.9, 18.4, 20.0, 25.7 and 30.4°C, respectively. As observed for *V. parahaemolyticus*, there was no lag phase and MPD depended on storage temperature. The observed MPD levels were between 7.1 log<sub>10</sub> CFU/g (30.4°C after 29 h) and 8.4 log<sub>10</sub> CFU/g (3.6°C after 289 h).



Count (●) in log<sub>10</sub> CFU/g and fitted curve (—).

**Figure 8.** Growth of total viable bacteria count in live Pacific oysters (3.6 - 30.4°C).

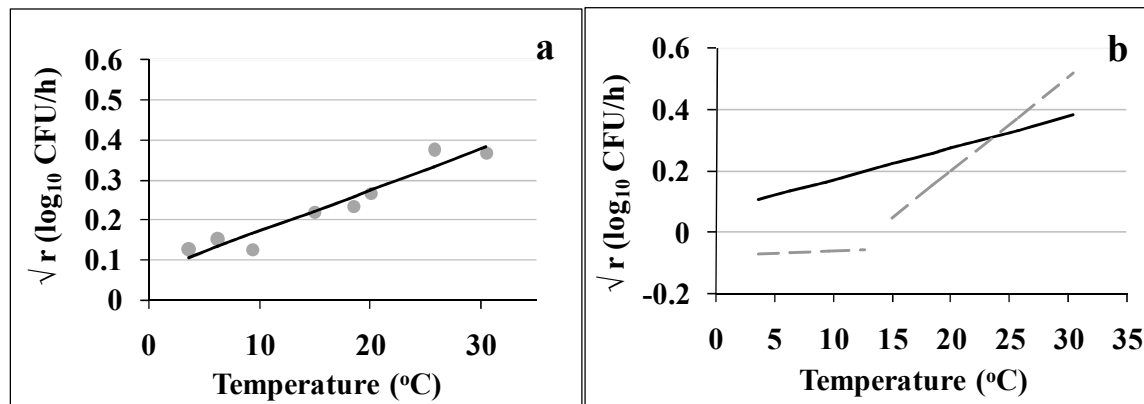
**Development and evaluation of a secondary model for total viable bacteria count in Pacific oysters.**

The square root of growth rate was plotted versus storage temperature and fitted with the square root model as shown in Figure 9a. The estimated values for parameters  $b$  and  $T_0$  were 0.0102 and -6.71, respectively (Equation 5). Goodness-of-fit comparing observed and predicted values showed a RMSE of 0.02 and a  $R^2$  of 0.93. The growth model was only applicable for a range of temperature from 3.6 to 30.4°C.

$$\sqrt{r} = 0.0102 \times (T + 6.71) \quad (5)$$

The secondary model (Equation 5) was evaluated against TVC growth rates at two temperatures for PO and five temperatures for SRO harvested in NSW. TVC multiplied in PO at 18 and 24.2°C at 0.012 and 0.026  $\log_{10}$  CFU/h, respectively. Populations increased from 5 to 6 logs MPN/g after 31.5 h storage at 24.2°C, and after 89.5 h storage at 18°C. TVC growth in SRO was only observed at 24.2°C. At this temperature, populations increased from 5  $\log_{10}$  CFU/g to 6.7  $\log_{10}$  CFU/g over 103.5 h showing a growth rate of 0.020  $\log_{10}$  CFU/h. Lower growth rates were observed compared to model predictions for PO and SRO from NSW at all tested storage temperatures.

TVC and *V. parahaemolyticus* models (Equations 3, 4 and 5) were compared as shown in Figure 9b. TVC rates were markedly greater than *V. parahaemolyticus* rates from approximately 4 to 23°C. However, *V. parahaemolyticus* growth rates exceeded that of TVC at temperatures greater than 23°C.



(a) Observed growth rates (●) and model (—). (b) Secondary models for: TVC (Equation 5) (—) and *V. parahaemolyticus* (Equation 3 and 4) (---).

**Figure 9.** Secondary model for total viable bacteria count (a) and its comparison to *V. parahaemolyticus* secondary models (b).

## 2.4 Discussion

Development of a predictive model for *V. parahaemolyticus* viability in oysters can improve risk management practices by identifying temperatures to control growth during post-harvest processing, storage and transport. Such intervention could decrease the risk of marketplace oysters with high levels of *V. parahaemolyticus* (22, 62) and also cases of infection from raw or undercooked oyster consumption (16).

Although predictive models have been previously developed for *V. parahaemolyticus* viability in broth systems (155, 233) and in food matrices (22, 231, 233), there is no such model for *V. parahaemolyticus* in live PO. In addition, little is known about *Vibrio* spp. viability in SRO.

In this study, data for model development were based on artificial inoculation of oysters by injection into the adductor muscle, a technique previously used to study summer mortalities for PO (83). Other studies have contaminated oysters by placing them in seawater aquaria inoculated with *V. parahaemolyticus* (205). It is possible that different routes of inoculation might affect the distribution of *V. parahaemolyticus* in oyster tissues. For example, in natural oysters *V. parahaemolyticus* and *V. vulnificus* appear to accumulate in higher densities in the digestive glands than in other tissues (211, 225). To take into account possible *V. parahaemolyticus* growth rates differences depending on the accumulated tissue, oysters with natural levels of the bacteria were used during model evaluation.

PO were injected with a cocktail of pathogenic and non-pathogenic *V. parahaemolyticus* strains to produce a model more representative of environmental situations. The use of fast-growing strains for modelling pathogenic *V. parahaemolyticus* has also been reported (155). The same strain may not display the fastest growth under all conditions (184), and a model based on a strain cocktail will generally result in more fail-safe predictions.

Levels of *V. parahaemolyticus* injected in PO were stable at 14.9°C during all experiments, and increased or decreased at other storage temperatures. Based on various reports, storage at 15°C may be close to the lower temperature boundary for *V. parahaemolyticus*. In a Korean oyster slurry, growth of *V. parahaemolyticus* was not observed at 15°C (233), whereas levels increased in artificially contaminated Zhe oysters (*Crassostrea plicatula*) at this temperature (205). Growth of *V. parahaemolyticus* at temperatures above 20°C has been previously reported for American oysters (51, 84) and in a Korean oyster slurry (233).

The estimated growth rate in American oysters stored at 26°C was 0.17 log/h (84): a slower growth rate was observed in this study for artificially contaminated PO.

Each storage condition tested presented a different MPD for *V. parahaemolyticus*. The highest observed was approximately 7.4 log<sub>10</sub> CFU/g at 25.7°C. This value exceeds the 6 log<sub>10</sub> CFU/g MPD reported in an artificial inoculated Korean oyster slurry at 20°C (233) and the 5.8 log<sub>10</sub> CFU/g for naturally occurring *V. parahaemolyticus* in American oysters stored at 26°C (84).

In addition to direct thermal effects, other factors that may influence *V. parahaemolyticus* growth include types and levels of competitive endogenous bacteria, host defence systems that vary by oyster species, and the possible release of antimicrobial factors when oyster tissue is homogenised (i.e. oyster slurry). Lag phase parameter was not considered for primary modelling. It is reasonable not to assume a lag phase when there are no adverse conditions of temperature, pH, water activity or nutrient availability that could stress bacteria and induce lag time (84), and it is a “fail-safe” approach.

Numerous studies have reported that *V. parahaemolyticus* is sensitive to and gradually inactivated by cold storage temperatures. Below 12.6°C, levels of injected *V. parahaemolyticus* in PO decreased. Similarly, *V. parahaemolyticus* growth was not observed at 10°C in a Korean oyster slurry and in American oysters (51, 233). In contrast, the minimum temperature for *V. parahaemolyticus* growth in broth has been reported to vary between 5°C (41) and 8.3°C (155). Differences in the minimum temperature for growth and survival of *V. parahaemolyticus* can be due to strain variation (41) and/or to the sample matrix (233). We observed a reduction of 2.5 log<sub>10</sub> CFU/g after 18 d storage at



3.6°C which is higher than the 0.8 log<sub>10</sub> CFU/g reduction for natural populations in American oysters after 14 - 17 d storage at 3°C (84).

The square root model (Equation 3) described slower growth than for previous *V. parahaemolyticus* models, with the exception of a Korean oyster slurry (Figure 7b).

Differences among these studies can be due to different food matrices, strain variability and/or the role of oyster host defences.

Oysters containing natural populations of *V. parahaemolyticus* were used for model evaluation studies. Levels of *V. parahaemolyticus* in PO and SRO during summer and early autumn were in the range of 2.4 to 4.0 log<sub>10</sub> MPN/g after non-refrigerated shipment of approximately 26 h. These levels are in agreement with reported concentrations of *V. parahaemolyticus* ranging from 0.4 MPN/g to 4.4 log<sub>10</sub> MPN/g, and a median of 3 log<sub>10</sub> MPN/g, in purified live New South Wales SRO sampled at wholesale (73). A similar *V. parahaemolyticus* mean level of 2.4 log<sub>10</sub> CFU/g was found in purified live SRO shipped from New South Wales to Tasmania in April 2002 (125). These average reported *V. parahaemolyticus* levels are also similar to levels of 2 to 3 log<sub>10</sub> CFU/g found in American oysters during summer (63).

Growth of *V. parahaemolyticus* in PO from New South Wales was only observed at 23 and 28°C, and at slower rates than those predicted. The observed could be due to variation in levels and types of competitive flora among different oyster growing regions, and/or differences in bacterial strains. These two challenge experiments were performed at SARDI laboratories. Possibly different shipment time and temperature from New South

Wales to SARDI laboratories compared to those in Tasmania may have caused changes in profiles of background bacteria.

Interestingly, SRO did not allow *V. parahaemolyticus* growth at any storage temperature. This agrees with other reported studies in which *V. parahaemolyticus* counts did not increase significantly when SRO were stored at 30°C for as long as 7 d (73). Such resistance to *V. parahaemolyticus* growth may indicate that SRO have enhanced host defences. It has been noted that SRO are unique in been able to survive high air temperature up to 36°C and a wide salinity range of 1.5 to 5.5% (164).

Reports show that *V. parahaemolyticus* pathogenic strains have longer lag times and slower growth rates than non-pathogenic strains (233), and that levels of pathogenic strains are generally several logs lower than total *V. parahaemolyticus* in harvested oysters (62, 63, 176). We used a combination of strains that possessed the *tdh* and *trh* genes as virulence markers as well as non-pathogenic strains. Criticisms are that these major virulence factors may not fully account for representation of all clinical strains (102, 222). Specifically, a heat-labile protein (protease A) produced by a clinical *V. parahaemolyticus* *tdh*-, *trh*- strain has been identified as a potential virulent factor (123). Furthermore, recent outbreaks have been related to a non-pandemic *tdh*-, *trh*-negative strain (82).

Levels of *V. parahaemolyticus* in oysters are commonly measured by the MPN method described in standard methods for the examination of foods (AS/NZS 1766.2.9:1997, ISO/TS 21872-1:2007). However, this method is time-consuming, labour-intensive, and not amenable to studies where numerous time intervals are necessary to build a robust model. For model development, we used the TCBS direct-plating method because

*V. parahaemolyticus* cells were injected into oysters at levels well above the low background (<600 CFU/g) levels of indigenous *Vibrio* spp. When background *Vibrio* spp. were detected in controls, it was after exponential growth for the injected *V. parahaemolyticus* strains. In addition, direct-plating on TCBS and conventional MPN method as described in Kaysner et al. (115) were compared at six time intervals during the model production. *V. parahaemolyticus* enumeration was not significantly different (Student's *t* test,  $P > 0.05$ ) (Appendix A2). However, if differences would have occurred between both methods, they would be consistent through all measurements and would not affect the growth rates value.

During evaluation studies, the MPN-PCR format was preferred over plating enrichments on TCBS because the former method was not influenced by non-*V. parahaemolyticus* bacteria that can also grow on TCBS and interfere with detection (70). The MPN-PCR method has been evaluated successfully for *V. parahaemolyticus* detection and enumeration in seafood samples (35). The sensitivity of the MPN-PCR method used in this study was examined and it was found to detect the *tlh* gene when enrichment broth was seeded with as few as 1.3 cells/ml (Figure 3d).

The kinetics of TVC growth were studied. There was an approximate increase of 2.5 and 3  $\log_{10}$  CFU/g in TVC for PO stored at 20.0°C for 3 d and at 6.2°C for 10 d, respectively. TVC levels have also been measured in American oysters, an increase of approximately 3 logs were observed after storage at 7 and 21°C for 10 d (129) and an increase of approximately 1  $\log_{10}$  was observed after storage at 22°C for 3 d (51). Differences among studies could be due different oyster species, media composition (e.g. NaCl level), as well

as incubation temperature which can select for the growth of different indigenous bacterial species.

The secondary model for TVC (Equation 5) predicted higher growth rates than those observed for PO and SRO from New South Wales. Possible reasons may include that the growing waters in New South Wales select for a different type of bacterial flora, as mentioned in previous similar observations (129), showing different growth rates compared to bacteria found in the relatively cooler waters in Tasmania. Also, seawater injection may have injured oyster tissues causing a release of nutrients, a change in oyster physiology and/or lower defence systems that resulted in higher bacterial growth. Another possibility is that shipment from New South Wales could have enhanced the growth of different bacteria. Moreover, bivalve feeding rate is temperature-dependent and oysters harvested from different locations and at different times may have had different bacterial species composition and concentrations.

Similar to *V. parahaemolyticus* validation studies, TVC rates differed markedly between PO and SRO. For example, TVC growth at 24.2°C was approximately 1.5 times slower in SRO than PO.

When the TVC model is compared to the *V. parahaemolyticus* model (Figure 9b), *V. parahaemolyticus* shows higher growth rates than TVC at temperatures above 21°C. Since sensory analyses were not performed in this study, it cannot be determined if *V. parahaemolyticus* would grow to high levels before consumers rejected the product. In the case of *V. vulnificus*, elevated levels in American oysters during storage trials were observed before 100% sensory rejection (129). Those authors suggested that simple

olfactory analysis of raw shell-stock may not be an adequate means to prevent oyster-associated *V. vulnificus* infections.

The *V. parahaemolyticus* mathematical models reported in this study can be used by regulatory agencies, oyster producers and consumers to minimize risk of illnesses, enhance product quality and manage supply chains. The models for *V. parahaemolyticus* viability (i.e. growth and inactivation) reduce uncertainty in the exposure model component of the VQRA, as growth rate was estimated from broth-based studies and extrapolated to oysters for only one temperature (17). Kinetic data for *V. parahaemolyticus* viability in PO and SRO generated during this study have been submitted to the United Nation's Food and Agriculture Organisation (FAO)/World Health Organisation (WHO) risk assessment group in response to a 2010 Call for Data. These data will be used to evaluate the validity of models used to help nations manage *Vibrio* spp. risk in foods.

The *V. parahaemolyticus* mathematical models reported in this study could also be integrated with remote-sensing technology. This technology uses satellites to measure the ocean radiation which can be empirically related to SST. The use of remote-sensing technology for prediction of incidence of *V. parahaemolyticus* was evaluated in American oysters (178) and presented a good correlation with direct measurements of *V. parahaemolyticus* densities in oysters at harvest.

With further refinement and validation, the model for TVC growth in PO could be used to manage oyster supply chains and identifying practices that can limit TVC growth (i.e. reduce spoilage). The levels of TVC that correlate with oyster spoilage and other organoleptic properties have not yet been determined.

This study reports the development and evaluation of models for *V. parahaemolyticus* and TVC viability in live Pacific oyster (*C. gigas*) based on the effect of post-harvest temperature in the range 3.6 to 30.4°C. This knowledge will be translated into tertiary models (computer software programs) that can be used by the oyster industry to optimize process conditions and reduce the risk of *V. parahaemolyticus* illness. The models are overly fail-safe for SRO, requiring a separate approach yet indicating that temperatures controls for this species could be different to manage *V. parahaemolyticus* risk.

### **3 – Evaluation of a software program for predicting *Vibrio parahaemolyticus* and total viable bacteria count levels in Pacific oysters (*Crassostrea gigas*) in simulated supply chain studies**

#### **3.1 Introduction**

The growth of *V. parahaemolyticus* and also spoilage bacteria can be controlled under proper temperature storage. However, an optimal cold supply chain for the entire distribution of oysters is difficult to achieve and inadequate temperature can occur (62, 132). For this reason, it is important to understand how bacteria can survive or multiply in the oyster from the farm to the consumer under dynamic temperature conditions.

Predictive microbiology can be used as a tool for supply chain management, and is based on mathematical models to estimate the responses of micro-organisms in previously studied environments. These mathematical models can be classified as primary, secondary and tertiary. The primary model describes the kinetics of bacterial viability under a given set of conditions. Kinetic parameters defined by the primary model are then described as a function of environmental factors in secondary models. Lastly, tertiary models integrate primary and secondary models in user-friendly software programs (67, 229).

The development of user-friendly application software provides many different users (e.g. food industry, risk assessors, food microbiologists) with greater access to the applications of mathematical models. Users define environmental parameter inputs and then easily

observe model outputs in graphic formats, allowing people without insight in mathematics to obtain useful information rapidly (38, 55, 210). Some examples of programs which can be used to predict the growth and inactivation of foodborne bacteria, primarily pathogens, under various environmental conditions are the Growth Predictor

(<http://www.ifr.ac.uk/safety/growthpredictor/> [accessed 30/05/11]) developed by Food Standards Agency and the Institute of Food Research in the UK; and the Pathogen Modelling Program (<http://pmp.arserrc.gov/PMPOnline.aspx> [accessed 30/05/11]) initiated by the US Department of Agriculture in the 1990s (38). Some examples of more specific programs are the SSSP (<http://sssp.dtuqua.dk/> [accessed 30/05/11]) which was developed to predict and illustrate the effect of constant and fluctuating temperatures on growth of SSO and on remaining shelf-life of different seafood products (56); and the Refrigeration Index (<http://www.foodsafetycentre.com.au/refrigerationindex.php> [accessed 30/05/11]) which predicts the growth of *E. coli* on meat from cooling profiles measured by electronic temperature loggers (147). Also, a program for the prediction of *V. parahaemolyticus* growth in broth systems at different temperatures in the range 15 - 30°C, pH and salt concentrations has been reported by Fujikawa et al. (80).

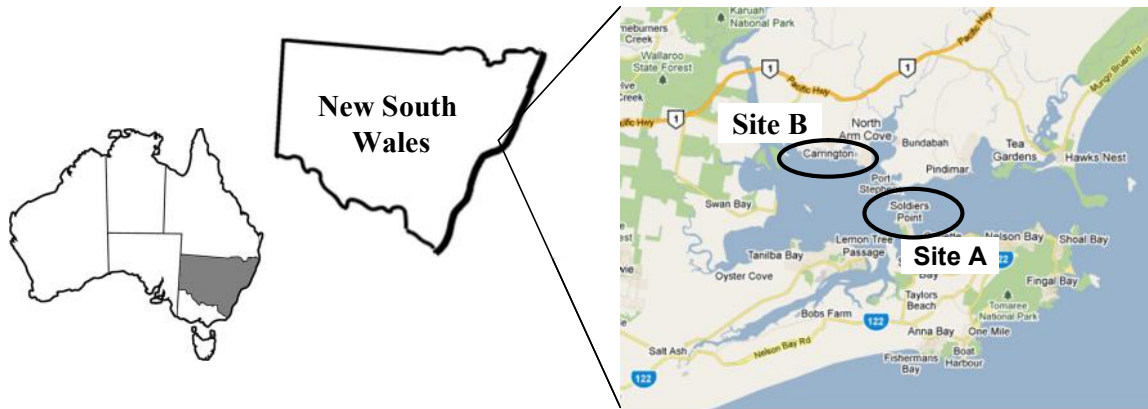
As with many other programs available for food safety management, predictions are specific to certain bacterial strains and environments that were used to generate the models. A drawback to the application of some predictive programs to food systems is a lack of validation studies measuring the performance of the model in a defined food matrix. This is an essential step to be performed before predictive models can be applied practically with confidence (149).



A program to predict the viability of *V. parahaemolyticus* in live PO over a wide range of storage dynamic temperatures would be beneficial to the oyster industry. For this reason, the *V. parahaemolyticus* and TVC predictive models for PO reported in Chapter 2 were used to develop a tertiary model. In this study, the performance of the program was evaluated with simulated oyster supply chain scenarios. In the evaluation, natural populations of *V. parahaemolyticus* in oysters exposed to fluctuating temperatures were measured at the beginning and at the end of supply chains. The program provides an important cold supply chain management tool to ensure the bacteriological quality and safety of oysters.

## 3.2 Materials and Methods

**Oyster samples.** Two batches of PO were harvested by two different commercial growers located in Soldiers Point (site A) and Carrington (Site B), both in New South Wales the 18th May 2010 (Figure 10). Following collection, oysters were placed in coolers and transported within 2 h to the Port Stephens Department of Primary Industries (DPI) laboratories, New South Wales. Oysters were washed with tap water to remove excess mud on shells and processed within 2 h. Salinity and SST data from the harvest area were obtained from oyster growers. A total of 175 oysters were used for experiments.



**Figure 10.** Oyster harvest sites.

**Simulated supply chain conditions.** Two commercial supply chain scenarios were designed to represent short and long refrigerated transport ( $<10^{\circ}\text{C}$  for  $72 \pm 2$  h or  $212 \pm 2$  h). A third scenario depicted overt thermal abuse ( $\sim 20^{\circ}\text{C}$  for  $70 \pm 2$  h).

Oysters from site A and B were separated in three and two different batches, respectively. One batch from each site was used for long refrigerated transport simulation and another for thermal abuse simulation. For the short refrigerated transport simulation, only a batch from site A was used.

To simulate supply chains, oysters were stacked in 2-3 layers in open plastic containers and stored in two different incubators set at temperature  $<10^{\circ}\text{C}$  for the refrigerated and  $\sim 20^{\circ}\text{C}$  for the non-refrigerated simulated supply chains. At selected times, oysters were removed from incubators, packed in coolers containing ice (ice packs were placed at the bottom of the cooler and oysters were wrapped in newspaper to avoid direct contact with ice) in the case of cold transport and without ice for the thermal abuse scenario. A total of five boxes were sent by overnight courier to the University of Tasmania food microbiology

laboratory in Hobart, Tasmania. After arrival, boxes were stored in a cold room set at 4°C (for the refrigerated scenario) or at room temperature ~20°C (for the non-refrigerated scenario) overnight and tested the following morning. The temperature profile was monitored by placing temperature data loggers (iButton<sup>®</sup>, Maxim Integrated Products, Inc., Sunnyvale, CA) among the oysters. The resolution of the temperature logger was 0.5°C.

**Bacterial enumeration.** Endogenous *V. parahaemolyticus* and TVC were enumerated using the MPN-PCR protocol and MA direct-plating, respectively, as described in Chapter 2 (section 2.2). A slight modification of the protocol was done for measuring *V. parahaemolyticus* and TVC levels prior to the simulated commercial shipment to the University of Tasmania. Specifically, a blender (Woolworths hand blender XB986, NSW, Australia) instead a stomacher machine was used to prepare oyster homogenate at the Port Stephens DPI laboratory.

*V. parahaemolyticus* and TVC were enumerated before (at the Port Stephens DPI laboratory) and after supply chain simulation (at the University of Tasmania laboratory). Two separate samples of 10 oysters were analysed at each sampling interval for each batch. The mean  $\pm$  SD for *V. parahaemolyticus* and TVC were measured. Mean values were transformed to log<sub>10</sub> values and the change in bacterial level calculated.

**Evaluation of the tertiary model.** The tertiary model used was based on the validated secondary models for *V. parahaemolyticus* and TVC in live Australian PO (Equations 3, 4

and 5) reported in Chapter 2 and summarised in Table 9. The maximum levels for *V. parahaemolyticus* and TVC were set to 7.4 MPN/g and 8.4 log<sub>10</sub> or CFU/g, based on the highest MPD observed during kinetic studies in Chapter 2 (section 2.3).

**Table 9.** Secondary models for *V. parahaemolyticus* and total viable bacteria count in Pacific oysters

Micro-organism/s	Type	Equation
<i>V. parahaemolyticus</i>	Growth	$r \text{ (log/h)} = [0.0303 \times (T - 13.37)]^2$
<i>V. parahaemolyticus</i>	Inactivation	$r \text{ (-log/h)} = -\exp [\ln 1.81 \times 10^{-9} + 4131.2 \times (1 / (T+273.15))]$
Total viable bacteria count	Growth	$r \text{ (log/h)} = [0.0102 \times (T + 6.71)]^2$

Source data: (Chapter 2: equations 3, 4 and 5).

The initial level of *V. parahaemolyticus* and TVC before shipment, and the time-temperature profile from the data loggers were set in the “Logger input” spreadsheet (Figure 11).

### DATA ENTRY

*If known, enter initial count (logCFU/g) in the spaces below. If not known, leave blank, or enter the harvest water temperature in the sheet "Start Here"*

***V. parahaemolyticus* (logCFU/g)** 0.68

**Total Viable Count (logCFU/g)** 4.54

(From "Start Here" or default value)

**Enter time and date that logging started, below:**

Start Date (dd/mm/yyyy) 4/05/2010

Start time (hh:mm) 15:30

**Enter the time interval between temperature readings below**

120 (minutes)

(n.b. model is only accurate for temperatures 3.5-30.5°C)

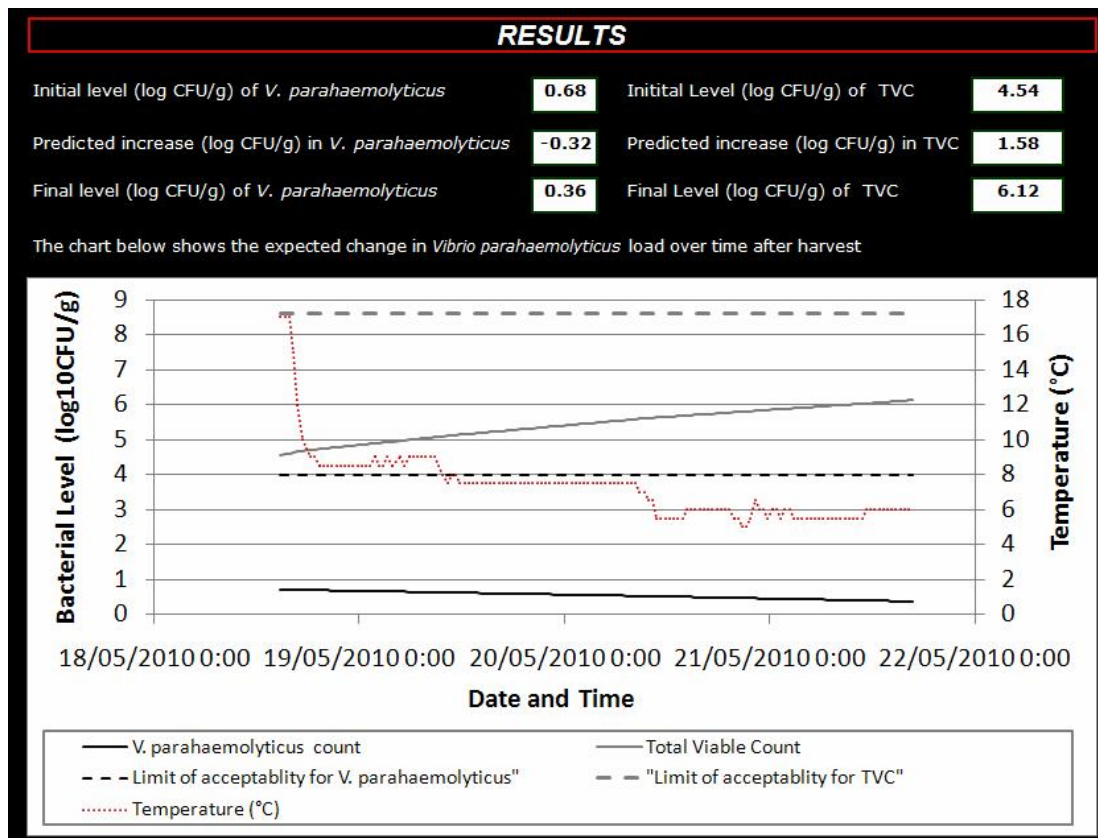
**Cut-and-Paste logged temperature (°C) data in the white cells below:**

18/05/2010 14:45	17
18/05/2010 15:15	17
18/05/2010 15:45	17
18/05/2010 16:15	15
18/05/2010 16:45	12
18/05/2010 17:15	10
18/05/2010 17:45	9.5
18/05/2010 18:15	9
18/05/2010 18:45	9
18/05/2010 19:15	8.5
18/05/2010 19:45	8.5
18/05/2010 20:15	8.5
18/05/2010 20:45	8.5

Initial bacterial data and a sample of the time-temperature profile from loggers for the short refrigerated supply chain simulation for oysters harvested at Site A.

**Figure 11.** Example of data input for the tertiary model.

Predicted *V. parahaemolyticus* and TVC final levels as a function of the supply chain temperature profile were derived from output section of the spreadsheet (Figure 12).



Outputs for the short-refrigerated supply chain simulation for oysters harvested at Site A.

**Figure 12.** Example of outputs from the tertiary model.

The observed and predicted growth rate for *V. parahaemolyticus* and TVC in each shipment were used to calculate the bias and accuracy factors (194). Growth rate was calculated as follows:

$$r = (\log_{10} N_{\text{final}} - \log_{10} N_{\text{initial}}) / t_{\text{sc}} \quad (6)$$

where  $N_{\text{final}}$  and  $N_{\text{initial}}$  are the final and initial bacterial level and  $t_{\text{sc}}$  the time (h) for a determinate supply chain simulation.

The bias and accuracy factors are indices that provide an objective summary of model performance. The bias factor is the average ratio of the predicted and observed values. A bias factor above one is obtained when there is over-estimation, while a value below one shows that the model is not safe and under-predicts. The accuracy factor is used for the same purpose but is an absolute value that avoids the effect of opposing estimations.

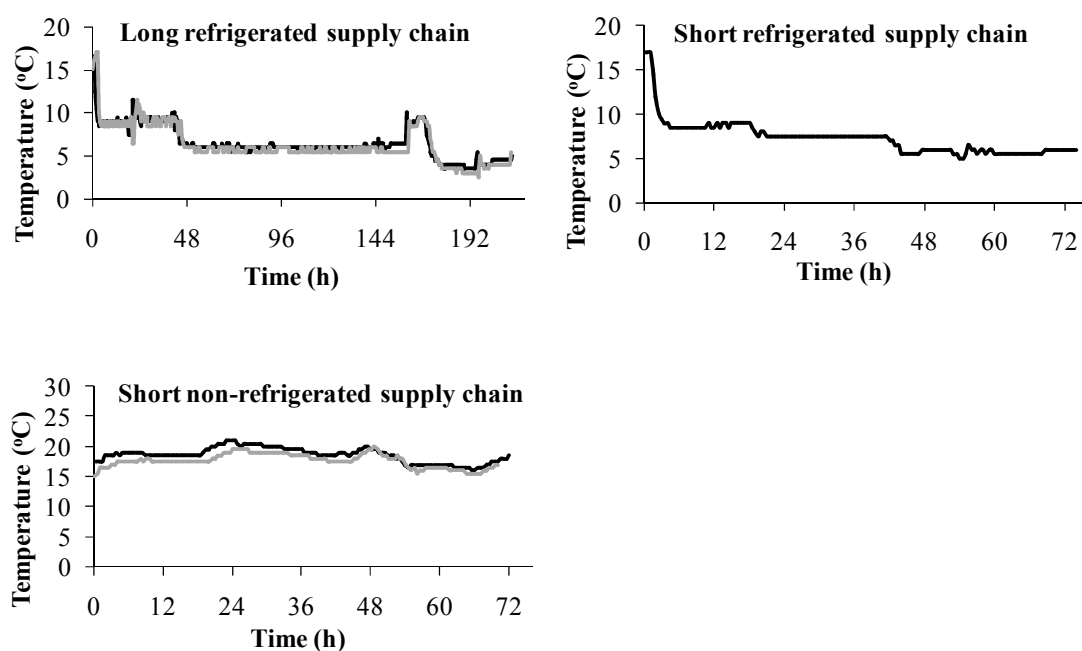
### 3.3. Results

**Environmental conditions and bacterial enumeration at harvest.** The salinity at the harvest site was 3.1 and 3.3‰ for sites A and B, respectively. Corresponding SST values were 15.6 and 17°C, respectively.

*V. parahaemolyticus* was detected in three of the four batches. The *V. parahaemolyticus* level in one sample from Site B was below the limit of detection (<0.3 MPN/g); the value was set to 0.3 to represent a worst-case scenario.

Initial mean levels of *V. parahaemolyticus* and TVC in oysters were 4.83 MPN/g and 4.54  $\log_{10}$  CFU/g for site A and 7.50 MPN/g (including the 0.3 MPN/g sample) and 4.26  $\log_{10}$  CFU/g for site B.

**Temperature profiles for supply chain simulations.** The recorded time and temperature for the different supply chains (long refrigerated, short refrigerated and short non-refrigerated) were pooled from the data loggers and are summarized in Figure 13. Total times were 213.5 and 212.5 h for site A and B long-refrigerated chain; 74 h for the site A short-refrigerated chain; and 72 and 70 h for the site A and B short non-refrigerated chain. Temperature during the refrigerated supply chain was  $<10^{\circ}\text{C}$ . The lowest temperature recorded was  $3^{\circ}\text{C}$  for site B and  $3.5^{\circ}\text{C}$  for site A for the long supply chain, and  $5.5^{\circ}\text{C}$  for the short supply chain from the site A. The range of temperature for the non-refrigerated supply chain was from 21 to  $16.5^{\circ}\text{C}$  for site A and  $19.5$  to  $17^{\circ}\text{C}$  for site B.



Oysters harvested in site A (—) and site B (---).

**Figure 13.** Temperature profiles for simulated supply chains.



**Bacterial levels in the long refrigerated supply chain simulations. *Vibrio***

*parahaemolyticus* levels in oysters decreased in both independent shipments for sites A and B. At the end of shipment, the mean level was 1.51 MPN/g after 213.5 h for site A and 0.64 MPN/g after 212.5 h for site B. This corresponded to a reduction of 0.50 log<sub>10</sub> MPN/g for site A and 1.08 log<sub>10</sub> MPN/g for site B (Table 10). The tertiary model predicted relatively similar reductions of 0.99 log<sub>10</sub> MPN/g for both sites.

**Table 10.** Change in *V. parahaemolyticus* levels for the long refrigerated supply chain

		Observed initial level		Observed final level		Observed change	Predicted final level	Predicted change
		MPN/g	log <sub>10</sub> (MPN/g)	MPN/g	log <sub>10</sub> (MPN/g)	log <sub>10</sub> (MPN/g)	log <sub>10</sub> (MPN/g)	log <sub>10</sub> (MPN/g)
Site A	S1	0.36		2.10				
	S2	9.30		0.92				
	Mean	4.83	0.68	1.51	0.18	<b>-0.50</b>	-0.31	<b>-0.99</b>
	SD	6.32		0.83				
Site B	S1	15.00		0.92				
	S2	0.30		0.36				
	Mean	7.65	0.88	0.64	-0.19	<b>-1.08</b>	-0.11	<b>-0.99</b>
	SD	10.39		0.40				

Observed and predicted changes in bold; S: sample; level below limit of detection indicated in red.

In contrast to *V. parahaemolyticus*, TVC levels increased in both oyster batches. After shipment, the mean level was 6.71 log<sub>10</sub> for site A and 5.81 log<sub>10</sub> CFU/g for site B, corresponding to an increase of 2.17 log<sub>10</sub> and 1.55 log<sub>10</sub> CFU/g for sites A and B, respectively (Table 11). The predicted change was 3.86 log<sub>10</sub> and 3.84 log<sub>10</sub> CFU/g for sites A and B, respectively.

**Table 11.** Change in total viable bacteria count for the long refrigerated supply chain

		Observed initial level		Observed final level		Observed change	Predicted final level	Predicted change
		CFU/g	log <sub>10</sub> (CFU/g)	CFU/g	log <sub>10</sub> (CFU/g)	log <sub>10</sub> (CFU/g)	log <sub>10</sub> (CFU/g)	log <sub>10</sub> (CFU/g)
Site A	S1	22,900		5,620,000				
	S2	46,600		4,600,000				
	Mean	34,750	4.54	5,110,000	6.71	<b>2.17</b>	8.40	<b>3.86</b>
	SD	16,758		721,249				
Site B	S1	15,900		880,000				
	S2	20,300		418,000				
	Mean	18,100	4.26	649,000	5.81	<b>1.55</b>	8.10	<b>3.84</b>
	SD	3,111		326,683				

Observed and predicted changes in bold; S: sample.

**Bacterial levels in the short refrigerated supply chain simulation.** This scenario was only tested for oysters harvested in site A. The mean *V. parahaemolyticus* level in oysters after shipment was 2.3 MPN/g, corresponding to a decrease of 0.32 log<sub>10</sub> MPN/g (Table 12). The same reduction was predicted using by the tertiary model.

**Table 12.** Change in *V. parahaemolyticus* levels for the short refrigerated supply chain

		Observed initial level		Observed final level		Observed change	Predicted final level	Predicted change
		MPN/g	log <sub>10</sub> (MPN/g)	MPN/g	log <sub>10</sub> (MPN/g)	log <sub>10</sub> (MPN/g)	log <sub>10</sub> (MPN/g)	log <sub>10</sub> (MPN/g)
Site A	S1	0.36		2.30				
	S2	9.30		2.30				
	Mean	4.83	0.68	2.30	0.36	<b>-0.32</b>	0.36	<b>-0.32</b>
	SD	6.32		0.00				

Observed and predicted changes in bold; S: sample.

The mean TVC level in oysters at the end (74 h) of the supply chain simulation was 5.33 log<sub>10</sub> CFU/g, corresponding to an increase of 0.79 log<sub>10</sub> CFU/g (Table 13). The tertiary model predicted an increase of 1.54 log<sub>10</sub> CFU/g.

**Table 13.** Change in total viable bacteria levels for the short refrigerated supply chain

		Observed initial level		Observed final level		Observed change	Predicted final level	Predicted change
		CFU/g	log <sup>10</sup> (CFU/g)	CFU/g	log <sup>10</sup> (CFU/g)	log <sup>10</sup> (CFU/g)	log <sup>10</sup> (CFU/g)	log <sup>10</sup> (CFU/g)
Site A	S1	22,900		216,000				
	S2	46,600		215,000				
	Mean	34,750	4.54	215,500	5.33	<b>0.79</b>	6.08	<b>1.54</b>
	SD	16,758		707				

Observed and predicted changes in bold; S: sample.

### Bacterial levels in the short non-refrigerated supply chain simulations.

*Vibrio parahaemolyticus* grew in oysters that were stored and transported without refrigeration. The mean level in oysters was 6.55 and 22.65 MPN/g after 72 h and 70 h for sites A and B, respectively. This corresponded to a 0.13 and 0.47 log<sub>10</sub> MPN/g increase in *V. parahaemolyticus* levels for sites A and B, respectively (Table 14). The tertiary model predicted higher increases of 1.90 and 1.25 log<sub>10</sub> MPN/g for sites A and B, respectively.

**Table 14.** Change in *V. parahaemolyticus* levels for the short non-refrigerated supply chain

		Observed initial level		Observed final level		Observed change	Predicted final level	Predicted change
		MPN/g	log <sup>10</sup> (MPN/g)	MPN/g	log <sup>10</sup> (MPN/g)	log <sup>10</sup> (MPN/g)	log <sup>10</sup> (MPN/g)	log <sup>10</sup> (MPN/g)
Site A	S1	0.36		3.80				
	S2	9.30		9.30				
	Mean	4.83	0.68	6.55	0.82	<b>0.13</b>	2.58	<b>1.90</b>
	SD	6.32		3.89				
Site B	S1	15.00		2.30				
	S2	0.30		43.00				
	Mean	7.65	0.88	22.65	1.36	<b>0.47</b>	2.13	<b>1.25</b>
	SD	10.39		28.78				

Observed and predicted changes in bold; S: sample; level below limit of detection indicated in red.

The mean TVC level in oysters after shipment was 6.10 and 5.37 log<sub>10</sub> CFU/g for sites A and B, respectively, corresponding to an increase of 1.55 and 1.11 log<sub>10</sub> CFU/g for sites A and B, respectively (Table 15). The tertiary model predicted higher increases of 3.86 and 4.14 log<sub>10</sub> CFU/g for sites A and B, respectively.

**Table 15.** Change in total viable bacteria count for the short non-refrigerated supply chain

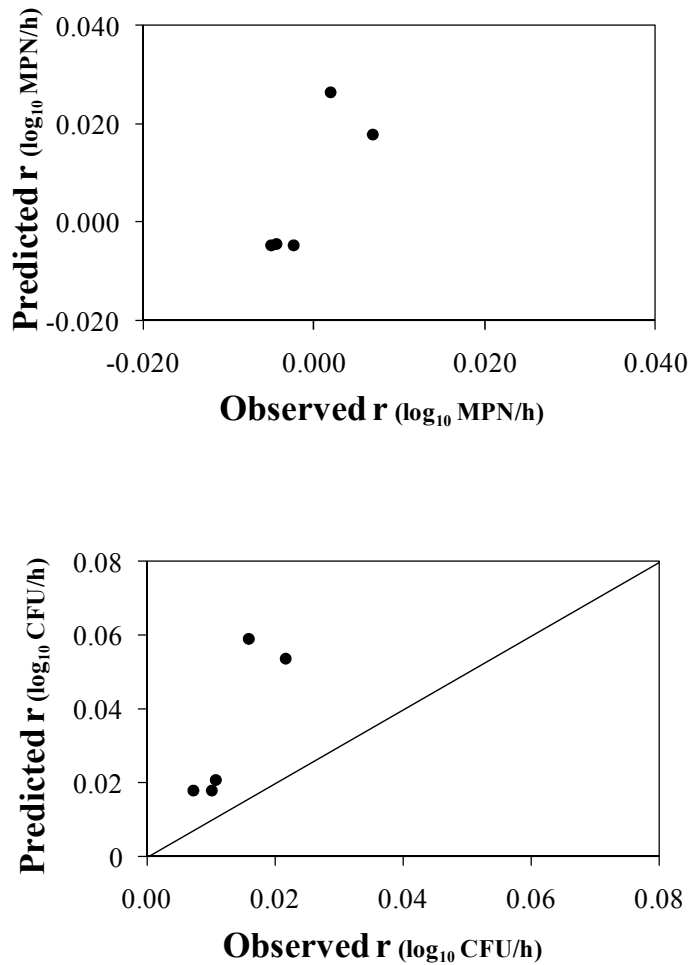
		Observed initial level		Observed final level		Observed change	Predicted final level	Predicted change
		CFU/g	log <sub>10</sub> (CFU/g)	CFU/g	log <sub>10</sub> (CFU/g)	log <sub>10</sub> (CFU/g)	log <sub>10</sub> (CFU/g)	log <sub>10</sub> (CFU/g)
Site A	S1	22,900		500,000				
	S2	46,600		1,990,000				
	Mean	34,750	4.54	1,245,000	6.10	<b>1.55</b>	8.40	<b>3.86</b>
	SD	16,758		1,053,589				
Site B	S1	15,900		264,000				
	S2	20,300		206,000				
	Mean	18,100	4.26	235,000	5.37	<b>1.11</b>	8.40	<b>4.14</b>
	SD	3,111		41,012				

Observed and predicted changes in bold; S: sample.

**Evaluation of the software.** When all supply chain scenarios were compared for predicted versus observed *V. parahaemolyticus* growth rate (Figure 14); two measurements showed similar estimations, two an over-estimation and one an under-estimation. For TVC, under-estimations were not observed and the five observed r were lower than the predicted.

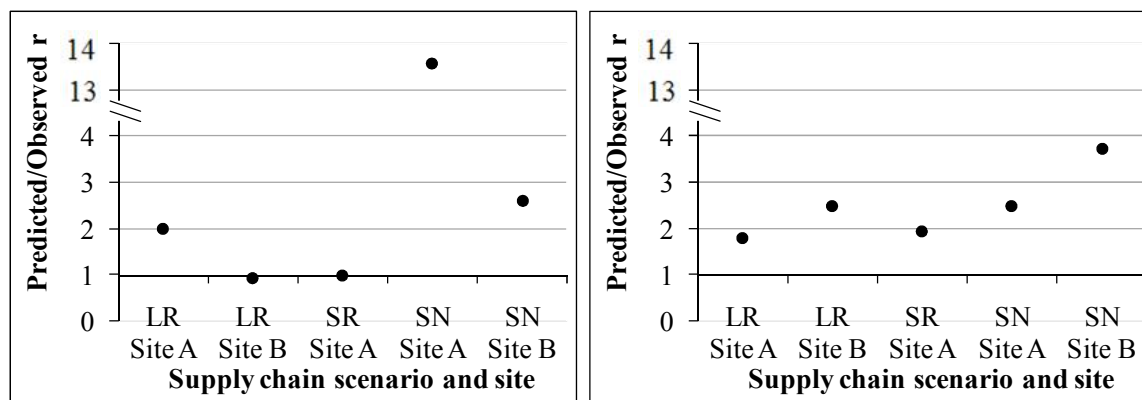
Taking into account all values, the tertiary model showed a bias factor of 2.30 for

*V. parahaemolyticus* and 2.40 for TVC. The accuracy factors were 2.38 (which included the under-estimation value) and 2.40 for *V. parahaemolyticus* and TVC, respectively.



**Figure 14.** Comparison of observed versus predicted growth rates for *V. parahaemolyticus* (up) and total viable bacteria count (down).

The ratio between predicted and observed growth rate for *V. parahaemolyticus* and TVC were plotted based on supply chain scenario and oyster harvest site (Figure 15). For *V. parahaemolyticus*, the highest over-prediction (ratio of 13.6) was for the short non-refrigerated supply chains in site A. In the case of TVC, the highest over-estimation (ratio of 3.7) was for the short non-refrigerated supply chain in site B.



Long refrigerated (LR), short refrigerated (SR) and short non-refrigerated (SN) supply chains.

**Figure 15.** Plots of the ratio between predicted and observed growth rates for *V. parahaemolyticus* (left) and total viable bacteria count (right) based on supply chain scenario and site.

### 3.4 Discussion

*Vibrio parahaemolyticus* naturally occurs in seawater, therefore pre-harvest management of levels that enter the supply chain is difficult to control. However, levels of *V. parahaemolyticus* post-harvest in oysters can be controlled with temperature. The purpose of this tertiary model was to provide a management tool to demonstrate how *V. parahaemolyticus* levels can be managed in oyster supply chains.

The advantages of tertiary models have been described for predictions of microbial viability in broth (38, 80), as well as in food products for controlling pathogenic bacterial growth in meat (147) and spoilage organisms in seafood (54). This software differs from other tools due to its specificity of usage.

The tertiary model was validated in two different commercial supply chain scenarios: refrigerated and non-refrigerated. The refrigerated shipment studies simulated the legal recommendations for PO in Australia (4) where PO must be kept at ambient temperatures <10°C after 24 h of harvest. However, deviations in temperature above 10°C during oyster distribution has been observed in the USA (50, 62) and Australia (132). Therefore, the tertiary model was also tested in a non-refrigerated shipment with temperatures in the range of 15 - 20°C. This non-refrigerated simulation also assisted in validating the growth models.

Initial mean *V. parahaemolyticus* levels in oysters were <1 log<sub>10</sub> MPN/g for the two different harvest sites. In the USA, detection of *V. parahaemolyticus* in oysters is associated with SST >14 - 15°C, however this is for a different oyster species, the American oyster (111, 176). In the present study, SST values were above that temperature range yet low *V. parahaemolyticus* levels were detected. This could indicate that the overall combination of environmental conditions in Australian waters, as well as a different oyster species, may present less favourable conditions for *V. parahaemolyticus*.

The overall performance of the model was found to be “fail-safe” with an over-estimation mean of 2.30 for *V. parahaemolyticus* and 2.40 for TVC growth, calculated by the bias factor index (194). However, a highly “fail-safe” model which may highly over-estimate the observed growth in a food product presents some downsides. It could cause unnecessary inspections or even recall of products from the market that may be perfectly safe.

The performance of tertiary models using the accuracy and bias factors was studied for *L. monocytogenes* growth in naturally contaminated cold-smoked salmon (57). In the previous study, over-estimation with bias factor from 1 to 5.2 was observed and the tertiary models could not be successfully validated. The same authors recommended that to improve the applicability of the models, studies need to include naturally contaminated products. We used a tertiary model developed with artificially inoculated oysters and validated with natural contaminated oysters, and also observed over-estimation. However, the degree of over-estimation is lower than if *V. parahaemolyticus* models developed in broth systems would have been used as discussed in Chapter 2.

Over-prediction of *V. parahaemolyticus* and TVC levels in oysters harvested in New South Wales was also observed during model evaluation and described in Chapter 2. Predictions may differ from observations due to variability among strains, interactions between micro-organisms and oyster host defence systems. Nevertheless, the overall performance of the model was “fail-safe” for predicting growth of *V. parahaemolyticus* in PO and would be a preferred public health tool.

Another possible cause for over-estimation could be that *V. parahaemolyticus* presented a lag phase in the oysters tested during supply chain studies. However, a *V. parahaemolyticus* lag phase in PO was not observed during kinetic studies in Chapter 2. Similarly, lag phase was not observed in naturally occurring *V. parahaemolyticus* in American oysters (84).

Currently, the predictive software only includes the effect of temperature in the growth of *V. parahaemolyticus* and TVC. The introduction of other factors may help to explain the



variability in bacterial changes observed among oysters shipped under similar conditions.

Microbial interactions can influence the growth of pathogenic micro-organisms, a phenomenon called the Jameson effect by Ross et al. (197). There are several predictive models which take into account microbial interactions. For example, the effect of lactic acid bacteria on the growth of *L. monocytogenes* has been modelled (153) and included in the SSSP predictive software.

Overall, predictions showed better agreement for the refrigerated compared to the non-refrigerated scenario. However, only five different real simulations were tested and a more extensive analysis of the performance of the model could change this observation.

The TVC model was added to the program as a potential tool for shelf-life prediction. However, accurate knowledge of TVC levels that correlate with organoleptic properties of PO is lacking and requires further investigation.

In conclusion, the tertiary model enables scientific knowledge about the viability of *V. parahaemolyticus* and TVC in PO to be transferred to risk managers via an Excel<sup>®</sup> interface. Future improvement in the software should include integrating a stochastic approach to incorporate uncertainties, as well as further studies about microbial indicators of oyster spoilage. A further evaluation of the program could include examining more simulated supply chains and measuring other factors which can affect *V. parahaemolyticus* growth.

## **4 – A cold chain management tool for the safety and quality of live oysters: a case study**

### **4.1 Introduction**

*Vibrio parahaemolyticus* can accumulate and multiply rapidly in oysters to levels that present a human health risk if supply chain temperature is not properly controlled (84). In addition, an increase in temperature could enhance the growth of spoilage bacteria affecting product shelf-life. Cold chain management is therefore necessary to ensure microbiological quality and safety.

In Australia, the temperature in which oysters need to be stored from production to consumption are regulated by the ASQAP (4). However, it is possible for temperature during storage, transport, retail display and at home to deviate from the recommended 10°C for PO. In fact, oysters exposed to temperatures exceeding this limit have been observed in distribution (62, 132) as well as in consumer refrigeration (8, 117, 137, 179). For this reason, tools to help food safety risk managers estimate the probability of occurrence of a hazard when temperature fluctuates are necessary.

Risk managers use QMRA as a tool to estimate risk quantitatively and protect public health (27). Predictive microbiology models can be used within QMRA to estimate changes in levels of microbial hazards in response to different environmental conditions (e.g. temperature) (146, 239). However, in QMRA, estimates of bacterial growth also need to be expressed in terms of probability (158). In this regard, simulation modelling software

packages (e.g. ModelRisk<sup>®</sup>) facilitate running Monte Carlo simulation, create suitable distributions, perform sensitivity analysis, and are often used for quantitative, stochastic, risk assessment (27, 66, 161).

Exposure assessment is the part of the QMRA in which the pathway by which the pathogen enters the food supply and subsequent changes in levels are analysed. The Modular Process Risk Model (MPRM) is a structured approach used to perform exposure assessment of bacteria in foods (162). It represents the food pathway as a chain of modules which are identified using microbial processes: growth, inactivation; and food handling processes: mixing, partitioning, cross-contamination and removal (159).

Supply chain management can also be improved with the use of traceability systems. The use of a combination of electronic traceability systems like RFID, Global Positioning System (GPS), internet and General Packet Radio Services (GPRS) networks can monitor distribution of the product as well as provide real-time safety and quality monitoring when used in combination with microbial modelling (79, 152, 235). The application of these systems would improve communication during supply chain, promote brand protection and avoid the arrival of low quality and/or unsafe oysters to the consumer.

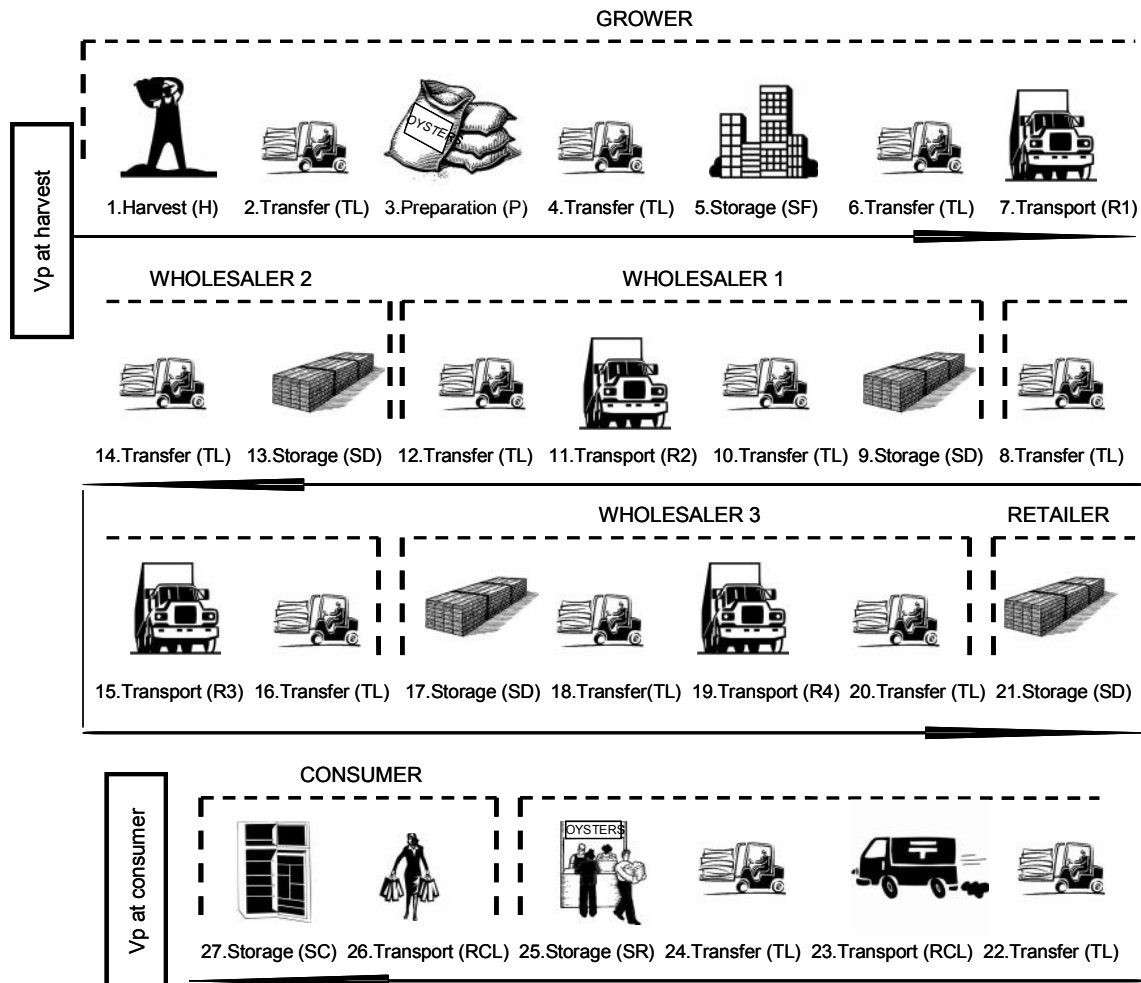
In this case study, ModelRisk<sup>®</sup> software was used to estimate the prevalence and concentration of total *V. parahaemolyticus* and TVC occurring (and accumulating) along two different oyster supply chains in summer and winter. The approach was to integrate the relevant supply chain operations (e.g. transport, storage, transfer) into a stochastic model that had input and output data described by distributions of parameters most likely observed in real supply chain scenarios, rather than using a single ‘best’ estimate. The

detailed case study demonstrated the use of predictive microbiology in a probabilistic modelling format which could be used as part of an exposure assessment for QMRA. It allowed sensitivity analyses to identify the critical operations. It also showed the advantage of the integration of predictive microbiology and stochastic modelling in traceability systems.

## 4.2 Materials and Methods

**Oyster supply chain structure.** The case study was reduced to an analysis of the oyster supply chain starting in Tasmania. The majority of Tasmanian oysters are distributed to domestic markets with the bulk of interstate sales going to Victoria and New South Wales.

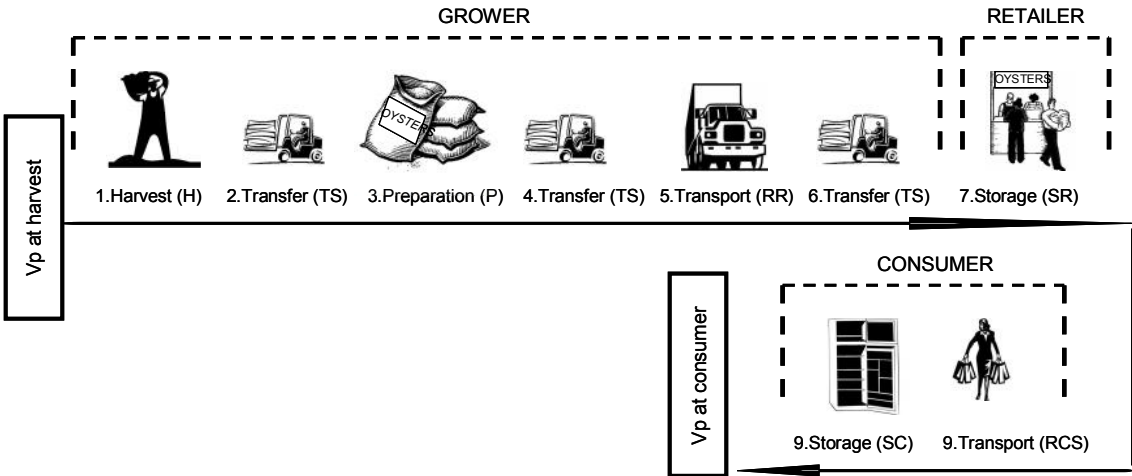
*Long supply chain case scenario.* This scenario represented an oyster supply chain from grower to consumer in which three wholesalers and one retailer were included (Figure 16). In this supply chain example, oyster growers from locations closer to Hobart sent product to a depot in Hobart. Subsequently, the product was transferred to refrigerated vehicles and transported to Melbourne via a vehicular ferry using the Bass Strait. Once in Melbourne, oysters were delivered to a depot and then transferred to a refrigerated vehicle for delivery to three wholesalers, until arrival at retail in Sydney from where the consumer purchased their oysters. The model included consumer transport and storage.



Source figures: (<http://office.microsoft.com/en-us/images/> [accessed 10/02/11]).

**Figure 16.** Structure for the long oyster supply chain from harvest to consumer storage.

*Short supply chain case scenario.* This second scenario represented an oyster supply chain from grower to consumer in which only one retailer was included (Figure 17). In this shipment, oyster growers from locations in Tasmania sent the product directly by refrigerated vehicles to Melbourne via a vehicular ferry using the Bass Strait. Once in Melbourne, oysters were delivered to retail outlets. Consumer transport and storage after retail was also modelled.



Source figures: (<http://office.microsoft.com/en-us/images/> [accessed 10/02/11]).

**Figure 17.** Structure for the short oyster supply chain from harvest to consumer storage.

**Oyster supply chain data.** Data from the operations of 12 commercial oyster supply chains from oyster farms in Tasmania and their time and temperature profile were evaluated by Madigan (132) for different seasons: four in spring, one in summer, five in autumn and two in winter as shown in Table 16.

**Table 16.** Summary of the oyster supply chain profiles for Tasmania

From	To	Date	Season	# Operations
Little Swanport, Tas	Melbourne	05/09/2008	Spring	7
Little Swanport, Tas	Melbourne	14/11/2008	Spring	6
Little Swanport, Tas	Melbourne	18/11/2008	Spring	6
Pittwater, Tas	Melbourne	02/09/2008	Spring	10
Blackmans Bay, Tas	Melbourne	03/12/2008	Summer	15
Blackmans Bay, Tas	Brisbane	17/04/2008	Autumn	18
Blackmans Bay, Tas	Sydney	15/04/2008	Autumn	19
Blackmans Bay, Tas	Sydney	17/04/2008	Autumn	19
St Helens, Tas	Melbourne	01/05/2008	Autumn	16
St Helens, Tas	Melbourne	01/05/2008	Autumn	10
Little Swanport, Tas	Melbourne	22/08/2008	Winter	7
Little Swanport, Tas	Melbourne	29/08/2008	Winter	7

Source data: Madigan (132).

Other different sources of information used in this study are described in the following paragraphs. When seasonal classification was needed, the data were grouped as follows:

December to February (summer) and June to August (winter).

*a) Seawater surface temperature.* Values for SST in selected oyster harvesting areas in Tasmania for the last ten years were obtained from the Tasmanian Shellfish Quality Assurance Program by Turnbull (2010, pers. comm.).

*b) Levels of total viable bacterial count after harvest.* Values for TVC were extracted from kinetic studies for the different PO batches harvested in Tasmania and New South Wales in 2008-2010 (Chapter 2, section 2.3).

*c) Transport times.* In the operations where information was required regarding transport times, the Google Maps webpage and the “get directions” application

(<http://maps.google.com.au/maps?hl=en&tab=wl>, [accessed 09/09/10]) was used. The time for the ferry trip was obtained from the time schedule of the Spirit of Tasmania (<http://www.spiritoftasmania.com.au/>, [accessed 10/09/10]).

*d) Air temperatures.* When data regarding air temperature were necessary, the average of maximum and minimum temperatures for the last ten years available in selected areas and months were obtained from the Bureau of Meteorology (BOM, <http://www.bom.gov.au/climate/data/>, [accessed 24/05/11]).

*e) Temperature at retail storage.* Information regarding the percentage of oyster lots stored at different temperatures was extracted from a market survey study performed in the USA (62). In the study, temperatures under which oysters were stored at retail were determined by measuring the ambient air temperature in the cooler.

*f) Time for consumer and retail transport.* Times were taken from a study (139) that reviewed time and distances from over 900 collection districts in Melbourne to major supermarkets.

*g) Temperatures for consumer storage.* Temperature data were extracted from a recent domestic refrigerator survey performed in New South Wales (8). Data used were extrapolated from maximum and minimum temperatures recorded in refrigerators less than five years old.

#### **Predictive models used for bacterial growth and inactivation.** Total

*V. parahaemolyticus* levels in oysters at the time of harvest were estimated depending on



SST value using the predictive model included in the VQRA (24). The model formula was simplified to:

$$\text{Log}_{10} V. \text{parahaemolyticus/g} = -1.03 + (0.12 \times \text{SST}) \quad (7)$$

Total *V. parahaemolyticus* and TVC levels in oysters in operations in which ambient air temperature and time were controlled were estimated using the secondary models for *V. parahaemolyticus* and TVC in live PO shown in Chapter 3 (Table 9).

**Data analysis.** The supply chain model combined the operations described for each case study and the microbial processes of growth and inactivation following the MPRM methodology (159). It was built as a spreadsheet model in Microsoft Excel<sup>®</sup> with the addition of the ModelRisk<sup>®</sup> version 3 (Vose, Belgium). The number of iterations per simulation was selected to 10,000. The RMSE value was used as a criterion for selection for the probability distribution, as a measure to evaluate the goodness-of-fit for each distribution model. The MPD values found during kinetic studies (Chapter 2, section 2.3) were used as a reference for the maximum bacteria levels. Specifically, the maximum limits were set to 7.4 log<sub>10</sub> CFU/g for *V. parahaemolyticus* and 8.4 log<sub>10</sub> CFU/g for TVC. Sensitivity analyses to compare the influence of the inputs on the output were graphically represented by tornado charts and spider plots. In the tornado charts, inputs are statistically ranked and plotted in descending order. The longer the bar, the greater the effect that input variable has on the model's output. The rank correlation can take values from -1 (when the input is large, the output is small), through 0 (no influence) to +1 (when the input is large, the output is also large). Spider plots show the variation of inputs on the x-axis against the

output in the y-axis looking at the cumulative percentiles. Those inputs presented as a horizontal line have little influence on the output. Spider plots have the advantage of giving a sensitivity scale in terms of the output value (instead of a correlation statistic number in tornado plots) and can describe better some significant relationships which would be missed in tornado plots (i.e. “U-shaped” relationships) (223, 224).

**Input data.** The data used as input for the supply chain case studies are described below according to the operation step. There were a total of 27 and nine operations for the long and the short supply chain, respectively. A summary for the input data and the distributions used to describe them are presented in Table 17 and 18.

**Table 17.** Input data and distributions used in the case study for the short supply chain

Operator	#	Operation	Input data	Data description			Distribution type	
				Units	Min	Max		Mean (SD)
Grower	1	Harvest	SST winter	°C	5.8	14.5	9.8 (1.4)	NormalFit(9.8,1.4)
			SST summer	°C	13.2	24.5	19.0 (2.3)	NormalFit(19.0,2.3)
			TVC	Log CFU/g	4.5	5.5	5.1 (0.3)	NormalFit(5.1,0.3,,Vose XBounds(4.4,5.6))
	2	Transfer	time	h	0.3	1.0	0.4 (0.2)	WeibullFit(2.7,0.5)
			Temperature winter	°C	3.5	16.0	9.5 (4.2)	Discrete
			Temperature summer	°C	6.0	27.1	16.3 (5.8)	Discrete
	3	Preparation at farm	time	h	0.7	5.0	3.6 (1.4)	PERT(0.7,4.3,5)
			Temperature winter	°C	3.5	14.8	9.5 (3.9)	Discrete
			Temperature summer	°C	9.0	23.0	16.1 (4.6)	Discrete
	4	Transfer	same for operation 2					
	5	Transport to retailer	time	h	10.8	16.1	14.3 (1.3)	NormalFit(14.3,1.3)
			Temperature winter	°C	5.0	9.5	6.2 (0.8)	PERT(5,6,9.5)
			Temperature summer	°C	6.5	14	7.4 (1.8)	PERT(6.5,6.5,14)
Retailer	6	Transfer	same for operation 2					
	7	Storage at retail	time	h				Normal(24.0,4.0)*
			Temperature	°C	5.0	17.0	11.3 (5.1)	Discrete
	Consumer	8	Transport at consumer	time	h	0.001	4.8	0.3 (0.4)
Temperature winter				°C	7.5	16.0	11.6 (4.0)	NormalFit(11.6,4.0)
Temperature summer				°C	14.4	27.1	21.1 (6.0)	NormalFit(21.1,6.0)
9		Storage at consumer	time	h				Normal(24.0,4.0)*
			Temperature	°C	-5.0	9.0	2.9 (3.5)	NormalFit(2.9,3.5)

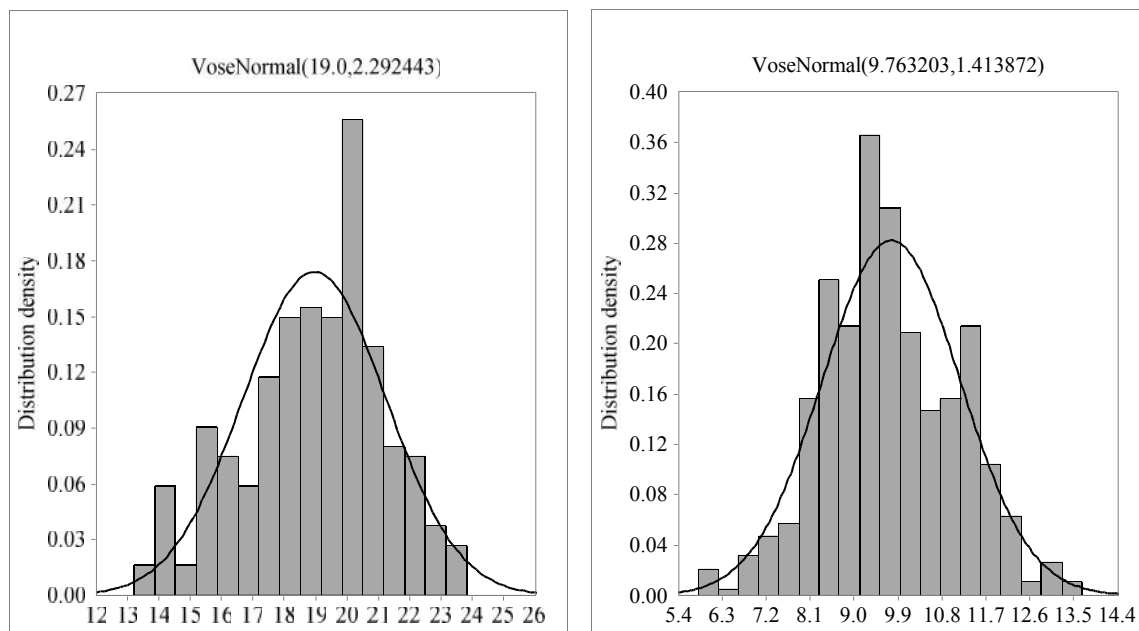
\* Assumed distributions, SD: standard deviation

**Table 18.** Input data and distributions used in the case study for the long supply chain

Operator	#	Operation	Input data	Data description				Distribution type	
				Units	Min	Max	Mean (SD)		
Grower	1	Harvest	SST winter	°C	5.8	14.5	9.8 (1.4)	NormalFit(9.8,1.4)	
			SST summer	°C	13.2	24.5	19.0 (2.3)	NormalFit(19.0,2.3)	
			TVC	Log CFU/g	4.5	5.5	5.1 (0.3)	NormalFit(5.1,0.3,,VoseXBounds(4.4,5.6))	
	2	Transfer	time	h	0.3	1.0	0.4 (0.2)	WeibullFit(2.7,0.5)	
			Temperature winter	°C	3.5	18.2	10.2 (4.4)	Discrete	
			Temperature summer	°C	9.0	28.2	18.3 (5.7)	Discrete	
	3	Preparation at farm	time	h	0.7	5.0	3.6 (1.4)	PERT(0.7,4.3,5)	
			Temperature winter	°C	3.5	14.8	9.5 (3.9)	Discrete	
			Temperature summer	°C	9.0	23.0	16.1 (4.6)	Discrete	
	4	Transfer	same for operation 2						
	5	Storage at farm	time	h	1.7	24.0	18.9 (8.5)	PERT(1.7,21.3,24.0)	
			Temperature	°C					Normal(10,2,,VoseXBounds(,14.5))*
	6	Transfer	same for operation 2						
	7	Transport to depot	time	h	0.4	2.3	1.2 (0.6)	WeibullFit(2.3,1.4)	
			Temperature winter	°C	5.0	9.5	6.2 (0.8)	PERT(5,6,9.5)	
			Temperature summer	°C	6.5	14.0	7.4 (1.8)	PERT(6.5,6.5,14)	
	8	Transfer	same for operation 2						
	9	Storage at depot	time	h	0.7	37	12.1 (14.6)	WeibullFit(0.8,10.2)	
			Temperature winter	°C					Normal(10,2,,VoseXBounds(,14.5))*
			Temperature summer	°C	6.5	13.5	11.3 (1.6)	NormalFit(11.3,1.6)	
Wholesaler A	10	Transfer	same for operation 2						
	11	Transport to wholesaler B	time	h				Normal(3.2,0.3)*	
			Temperature winter	°C	5.0	9.5	6.2 (0.8)	PERT(5,6,9.5)	
			Temperature summer	°C	6.5	14.0	7.4 (1.8)	PERT(6.5,6.5,14)	
	12	Transfer	same for operation 2						
13	Storage at depot	same for operation 9							
Wholesaler B	14	Transfer	same for operation 2						
	15	Transport to wholesaler C	time	h				Normal(10.5,0.3)*	
			Temperature winter	°C	5.0	9.5	6.2 (0.8)	PERT(5,6,9.5)	
			Temperature summer	°C	6.5	14.0	7.4 (1.8)	PERT(6.5,6.5,14)	
	16	Transfer	same for operation 2						
17	Storage at depot	same for operation 9							
Wholesaler C	18	Transfer	same for operation 2						
	19	Transport to retailer	time	h				Normal(9.9,0.5)*	
			Temperature winter	°C	5.0	9.5	6.2 (0.8)	PERT(5,6,9.5)	
			Temperature summer	°C	6.5	14.0	7.4 (1.8)	PERT(6.5,6.5,14)	
	20	Transfer	same for operation 2						
21	Storage at depot	same for operation 9							
Retailer	22	Transfer	same for operation 2						
	23	Transport at retail	time	h	0.001	4.8	0.3 (0.4)	WeibullFit(0.8,0.2)	
			Temperature winter	°C	8.5	18.2	13.5 (4.7)	NormalFit(13.5,4.7)	
			Temperature summer	°C	18.6	28.2	23.5 (4.4)	NormalFit(23.5,4.4)	
	24	Transfer	same for operation 2						
25	Storage at retail	time	h				Normal(24.0,4.0)*		
		Temperature	°C	5.0	17.0	11.3 (5.1)	Discrete		
Consumer	26	Transport at consumer	same for operation 23						
	27	Storage at consumer	time	h				Normal(24.0,4.0)*	
Temperature			°C	-5.0	9.0	2.9 (3.5)	Normal(2.9,3.5)		

\* Assumed distributions, SD: standard deviation

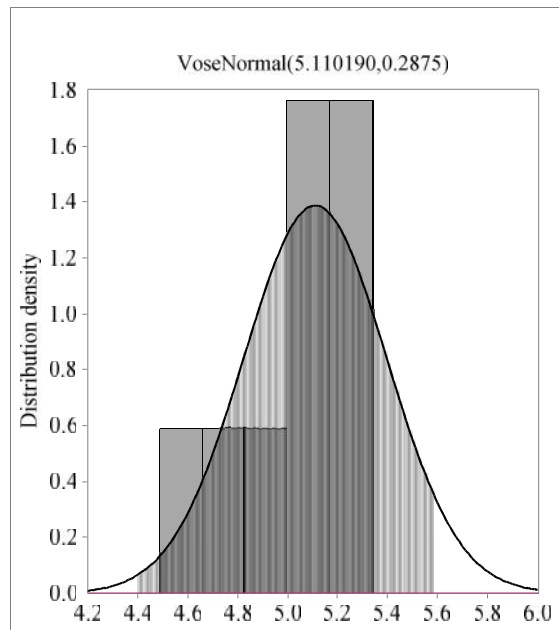
Oyster harvest. The values for SST in areas dedicated to oyster farming in Tasmania (Port Sorell, Little Swanport, Hastings Bay and PipeClay Lagoon) were classified for summer and winter periods. A total of 282 data points for seawater temperature at harvest were included for the summer distribution: the range was 13.2 to 24.5°C and the mean was  $19.0 \pm 2.3^\circ\text{C}$ . In winter, 462 data points were included showing a range of 5.8 to 14.5°C and a mean of  $9.8 \pm 1.4^\circ\text{C}$ . A normal distribution was chosen to fit the data because most values were near the average and the distribution was close to symmetrical (Figure 18).



Data source: Turnbull (2010, pers. comm.). X-axis: temperature ( $^\circ\text{C}$ )

**Figure 18.** Normal probability density distribution for seawater temperature at selected harvest areas in Tasmania for summer (right) and winter (left).

The levels of TVC at harvest were also included. There were ten data for TVC levels presenting a mean of  $5.1 \pm 0.3 \log_{10}$  CFU/g. The maximum level for TVC was  $5.5 \log_{10}$  CFU/g and the minimum  $4.5 \log_{10}$  CFU/g. A normal distribution was selected to fit the data but due to the small amount of samples the distribution was truncated to values between 4.4 and 5.6 as this was the range observed among the ten batches sampled (Figure 19).



Data source: Chapter 2, section 2.3. Shading indicates truncated values.

X-axis: total viable bacteria count in  $\log_{10}$  CFU/g.

**Figure 19.** Normal probability density distribution for total viable bacteria count in Pacific oysters after harvest in Tasmania and after shipment from New South Wales.

Transfer. There were 58 values available regarding times of loading and unloading oysters for transport. The time range during transfers varied from 0.3 to 1 h with a mean of  $0.4 \pm$

0.2 h. A Weibull distribution was fitted to the data with a shape parameter ( $\alpha$ ) of 2.7 indicating that was close to a normal distribution ( $\alpha \geq 3.25$ ) but values lower than 0.4 were more likely to occur.

Assuming that the transfer temperature would be similar to the ambient temperature, data for temperatures in areas where transfer operations were required (Hobart, Dover, Friendly Beaches, Devenport, Melbourne and Sydney) were included. Transfer temperatures were subclassified as TS for the short and TL for the long supply chain. TS differed to TL in not including temperature data in Sydney as the short supply chain finished in Melbourne.

For TS, a total of 32 observations ranging from 3.5 to 16.0°C and presenting a mean of  $9.5 \pm 4.2^\circ\text{C}$  were used for winter. A discrete distribution was used to give 50% weight to the only two experimental values (i.e. 5.5 and 7.5°C) from Madigan (132), while the 30 values from the BOM were weighted the other 50%. This may reflect that oysters are below the mean value (9.5°C) more often because they are still cold from the previous refrigeration step. For TS in summer, a total of 36 values in the range of 6.0 to 27.1°C and a mean of  $16.3 \pm 5.8^\circ\text{C}$  were included in a discrete distribution. As for winter, a 50% weight was given to the six experimental data values observed in summer by Madigan (132) and a 50% weight to the 30 values from the BOM.

The same assumption was used for the distributions in TL for data from Madigan (132) and BOM. TL also included six temperatures for Sydney. The mean value for the 38 data was  $10.2 \pm 4.4^\circ\text{C}$  (ranging 3.5 to 18.2°C) in winter and  $18.3 \pm 5.7^\circ\text{C}$  for the 42 data (ranging 9.0 to 28.2°C) in summer.

Preparation. The 12 times recorded during grading, counting and packing the oysters in the farm varied from 0.7 to 5 h with a mean of  $3.6 \pm 1.4$  h. A Pert distribution was selected with a mode of 4.3 as more of the half of the data was this value.

A total of seven temperatures for each season recorded during preparation were available from Madigan (132). Assuming that this operation is usually performed at ambient temperature, data for temperatures in areas dedicated to oyster farming in Tasmania (Hobart, Dover, Friendly Beaches and Devenport) were added. The data were organized using a discrete distribution, giving 50% weight to the seven experimental values observed by Madigan (132), while the remaining 24 values were weighted the other 50%. In total there were 31 data available for each season. In summer, the temperature range was from 9.0 to 23.0°C with a mean of  $16.1 \pm 4.6$ °C. In winter, temperature varied from 3.5 to 14.8 and had a mean value of  $9.5 \pm 3.9$ °C. This criterion was especially important for summer as recorded temperatures at farm were lower than the mean extracted from the BOM data. This assumption supported that during summer, oysters may be prepared in mornings or evenings when temperatures were lower.

Storage at farm. There were six values for times of oyster storage at the farm which varied from 1.7 to 24 h and showed an average of  $18.9 \pm 8.5$  h. As for preparation times, a Pert distribution was selected with a mode of 21.3 h as half of the data was this value and only one value was below 20 h.

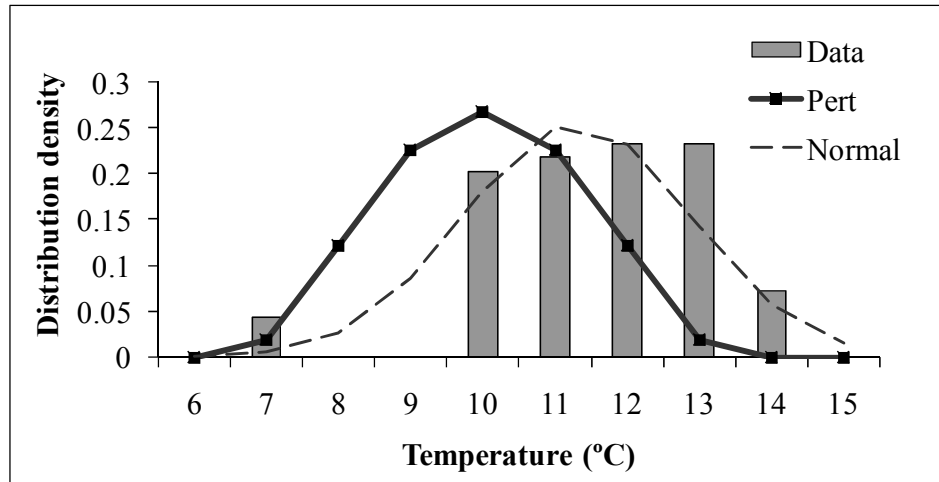
No data were available for storage temperature at the farm during winter. An assumption of temperatures in the range of 10°C was made. A normal distribution for a mean of  $10 \pm$

2°C, truncated in order to have only values below 14.5°C was used. The same assumption was used for storage temperature at farm during summer because the available five experimental values (in the range of 14 to 14.5°C) from Madigan (132) were not considered representative.

Storage at depot. A total of six values for times during storage at depot were used which were from 0.7 to 37 h with a mean of  $12.1 \pm 14.6$  h. A Weibull distribution was applied to the data with  $\alpha < 1$  to have more probabilities of sampling to lower values. This distribution reflects that long times (i.e. 37 and 22 h) were represented by only two values while the other four recorded storage times were below 7.7 h.

No data were available for temperature during storage at depot for winter and the same assumption as for farm storage was made. For summer, 69 values were available showing a range of 6.5 to 13.5°C and an average of  $11.3 \pm 1.6$ °C. Two different distributions were fitted to the data and compared (Figure 20). A Pert distribution with a mode value of 10°C had a RMSE of  $1.8 \times 10^{-4}$  while the RMSE for the normal distribution was  $3.9 \times 10^{-6}$ . Therefore, the normal distribution was preferred.





Data source: Madigan (132).

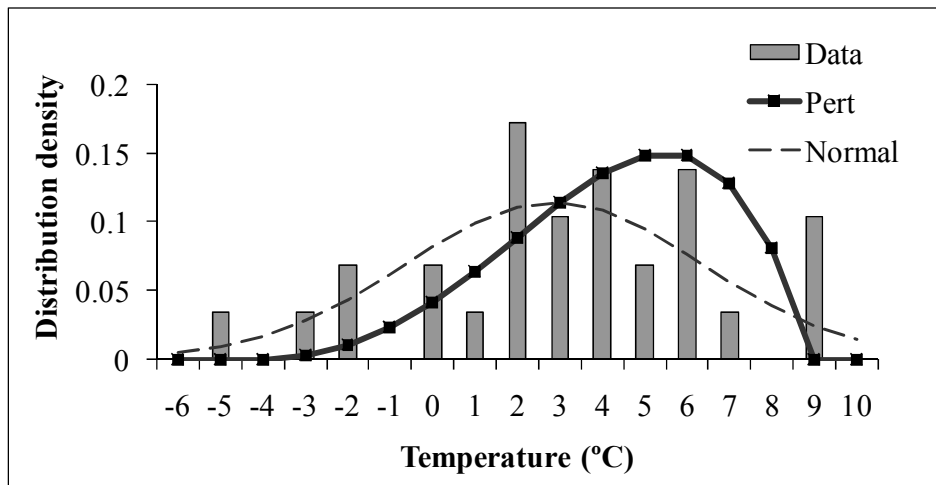
**Figure 20.** Comparison of Pert and normal probability density distribution for storage temperature at depot in summer.

Storage at retail. There were no experimental data regarding the time for this operation. It was assumed that storage times at retail were usually between 12 and 36 h, and a normal distribution was created with a mean value of 24 and standard deviation of 4 h.

Temperatures for this operation were not separated seasonally. A total of four different values: 5, 10, 13 and 17°C with different probability weights of 71, 14, 12 and 3% were used in a discrete distribution. The weights were selected to represent the proportion of oysters found at the different temperatures as reported in DePaola et al. (62).

Storage at consumer. The time for this operation was not available and the same assumption as for storage at retail was used here.

Regarding temperatures, there were 29 data which varied from -5 to 9°C and presented a mean value of  $2.9 \pm 3.5^\circ\text{C}$ . Model fitting was compared for a Pert distribution with a mode value of  $5.5^\circ\text{C}$  and a normal distribution (Figure 21). A RMSE value of  $7.8 \times 10^{-6}$  and  $2.6 \times 10^{-6}$  were observed for Pert and normal distributions, respectively. The normal distribution was chosen because it included the possibility of oysters being refrigerated at  $9^\circ\text{C}$  and presented slightly lower RMSE.



Data source: Anonymous (8).

**Figure 21.** Comparison of Pert and normal probability density distribution for storage temperature at consumer.

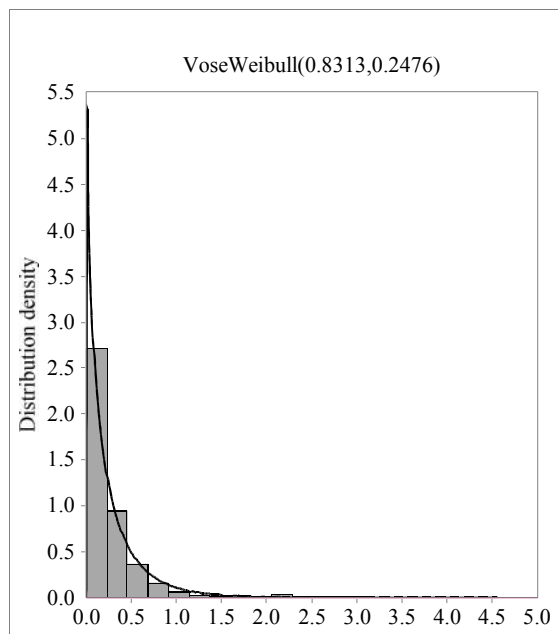
Transport from farm to retail. The time during transport for the long supply chain scenario was divided into four parts while the transport time for short supply chain case study was described by a single distribution. Temperature data used were the same for all the transport stages. For winter, 120 experimental included values from  $5$  to  $9.5^\circ\text{C}$  and had a mean of  $6.2 \pm 0.8^\circ\text{C}$ . For summer, the 60 experimental data varied from  $6.5$  to  $14^\circ\text{C}$  with a

mean of  $7.4 \pm 1.8^{\circ}\text{C}$ . Pert and normal distribution were compared for summer and winter data. In winter, the RMSE for Pert distribution and normal distribution were similar:  $5.2 \times 10^{-5}$  and  $4.8 \times 10^{-5}$ , respectively. In summer, the RMSE for Pert distribution was lower ( $5.1 \times 10^{-5}$ ) in comparison to the normal distribution ( $8.9 \times 10^{-4}$ ). The Pert distribution was selected for both seasons and mode values were 6 and 6.5 for winter and summer, respectively.

For the long supply chain, transport times between oyster growers close to Hobart (Dunalley, Dart Island, Port Arthur, Clifton Beach, Great Bay, Cloudy Bay, Deep Bay, Southport, Cockle creek, Midway Point and Dover) and Hobart depot were estimated. The eleven values ranged from 0.4 to 2.3 h with a mean of  $1.2 \pm 0.6$  h. Data were fitted using a Weibull distribution with a shape parameter of 2.3 to reflect that times lower than 1.2 were more likely to occur. The transport time from Hobart to Devonport, Devonport to Melbourne and Melbourne to Sydney were fitted in a normal distribution of  $3.2 \pm 0.3$  h,  $10.5 \pm 0.3$  h and  $9.9 \pm 0.5$  h, respectively.

Transport times for the short supply chain included all time combinations possible from different oyster farming areas in Tasmania (Montagu, Port Sorell, St Helens, Great Swanport, Little Swanport, Spring Bay, Dunalley, Dart Island, Port Arthur, Clifton Beach, Great Bay, Cloudy Bay, Deep Bay, Southport, Recherche Bay, Midway Point and Dover) to Melbourne. There were a total of 17 values ranging from 10.8 to 16.1 h and a mean of  $14.3 \pm 1.3$  h, data was fitted in a normal distribution.

Transport for retail or consumer. The times for consumer or retail transport were in the range of 0.001 to 4.8 h with an average of  $0.3 \pm 0.4$  h for the 985 data points examined. The data were observed to be concentrated in the lower values of time (Figure 22). A Weibull distribution was fitted to the data with  $\alpha < 1$  indicating that lower times of consumer or retail transport were more likely to occur.



Data source: Marquez et al. (139). X-axis: time (h).

**Figure 22.** Weibull probability distribution for times for consumer or retail transport.

Temperature during transport for retail or consumer was subclassified for the long and the short supply chains. A total of six temperature data for Sydney (long supply chain) and six temperature data for Melbourne (short supply chain) were described using a normal distribution.

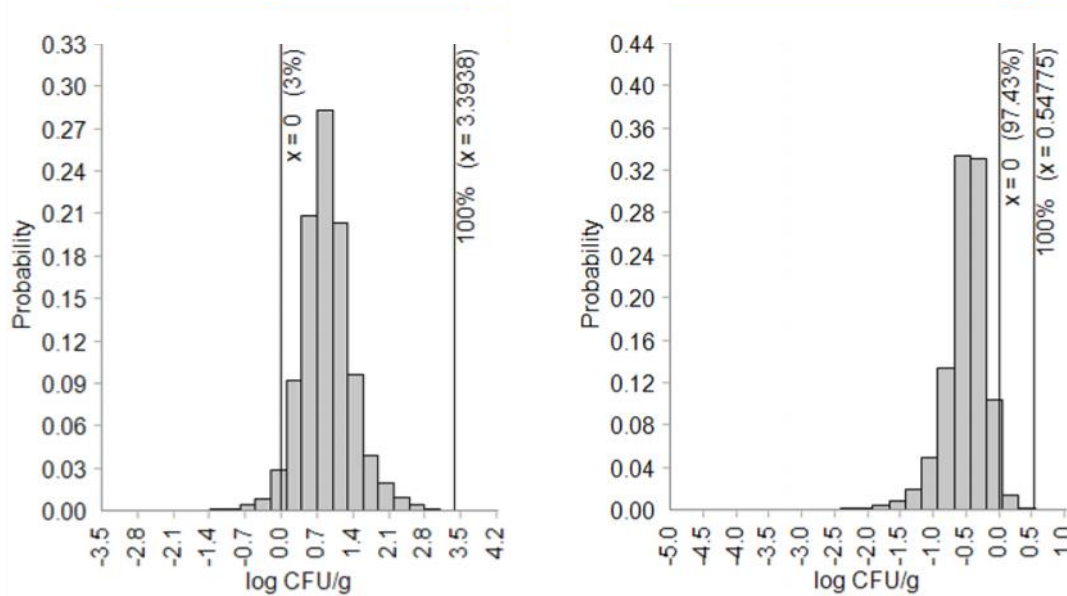
For the long supply chain, winter temperatures were in the range of 8.5 to 18.2°C with a mean of  $13.5 \pm 4.7^{\circ}\text{C}$ , and summer temperatures showed a mean of  $23.5 \pm 4.4^{\circ}\text{C}$  and varied from 18.6 to 28.2°C. For the short supply chain, the mean values were  $11.6 \pm 4.0^{\circ}\text{C}$  (ranging 7.5 to 16.0°C) and  $21.1 \pm 6.0^{\circ}\text{C}$  (ranging 14.4 to 27.1°C) for winter and summer, respectively.

### 4.3 Results

**Output data:** The output of the ModelRisk<sup>®</sup> simulation was the level of total *V. parahaemolyticus* and TVC in oysters after consumer storage. For each season (winter or summer) and supply chain structure (short or long), the distribution of total *V. parahaemolyticus* and TVC at consumer storage was obtained by using input data as described in Table 17 and 18. The lengths of the two different oyster supply chains predicted by the simulation were 2.5 and 6.0 d, for the short and long supply chains respectively.

*V. parahaemolyticus* after consumer storage. For the long supply chain scenario, estimated *V. parahaemolyticus* levels at consumption were generally higher in summer (with a mean of  $0.9 \log_{10}$  CFU/g) than winter (with a mean of  $-0.5 \log_{10}$  CFU/g). Levels of *V. parahaemolyticus* in oysters were predicted below  $3.4 \log_{10}$  CFU/g in summer and below  $0.5 \log_{10}$  CFU/g in winter (Figure 23). Only 3.0% of the oysters were predicted to

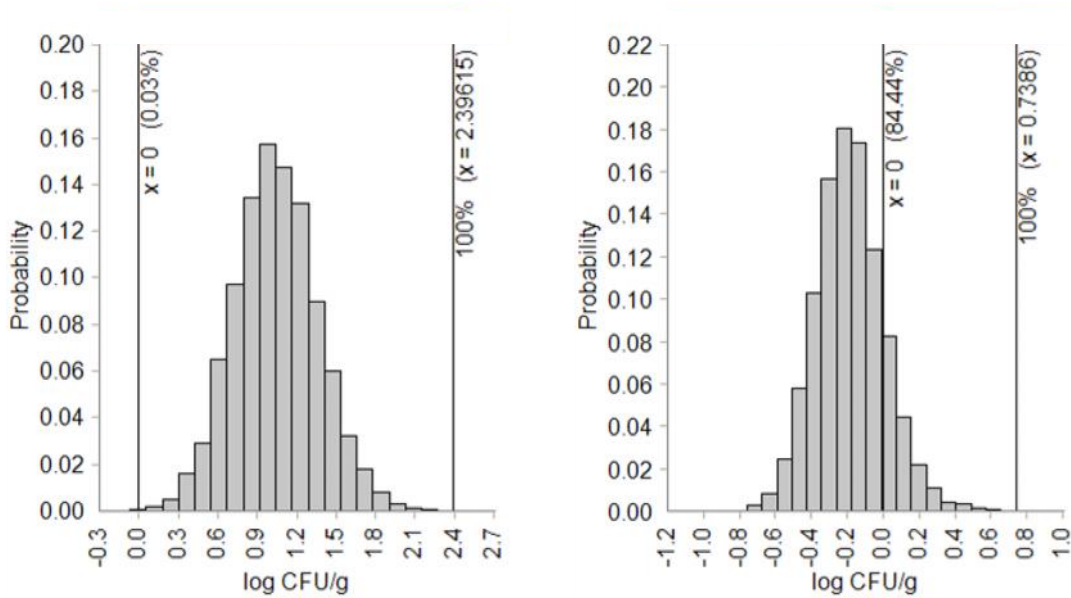
contain less than 1CFU/g ( $<0 \log_{10}$  CFU/g) *V. parahaemolyticus* during summer, compared to 97.43% in winter.



**Figure 23.** Predicted levels of total *V. parahaemolyticus* in oysters after consumer storage in the long supply chain in summer (left) and winter (right).

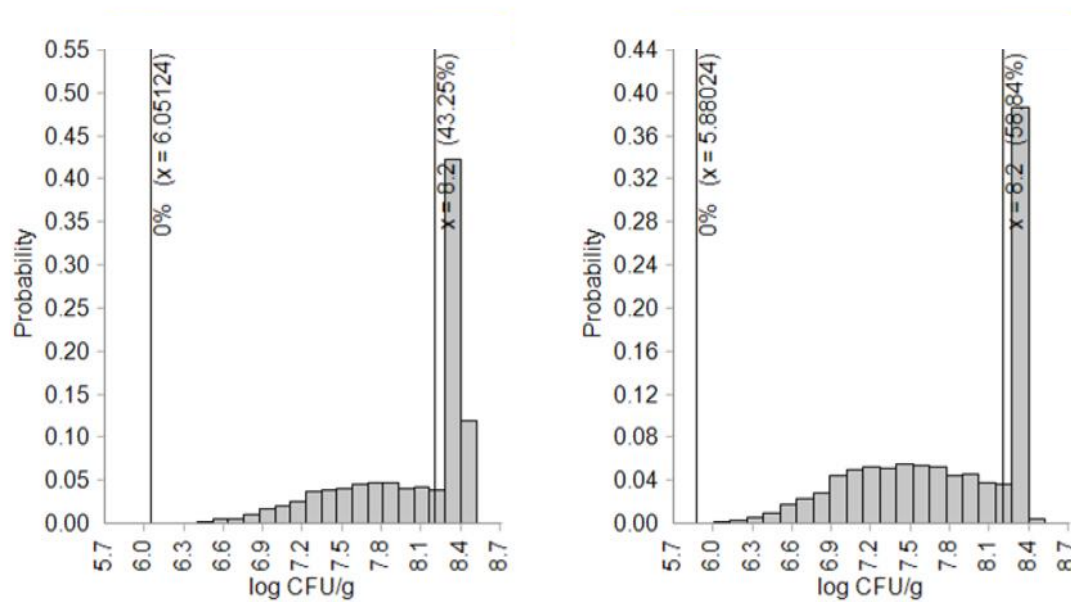
As observed for the long supply chain, levels of *V. parahaemolyticus* in the short supply chain were lower in winter (with a mean of  $-0.2 \log_{10}$  CFU/g) than summer (with a mean of  $1.0 \log_{10}$  CFU/g). The maximum levels predicted in oysters were lower for summer and slightly higher for winter in comparison to the long supply chain. All oysters were predicted to have levels of *V. parahaemolyticus* below  $2.4 \log_{10}$  CFU/g in summer and below  $0.7 \log_{10}$  CFU/g in winter (Figure 24). In summer, only 0.03% of oysters in the short

supply chain were predicted to have levels of *V. parahaemolyticus* below 1 CFU/g, while a much higher proportion (84.44%) of oysters were observed in winter.



**Figure 24.** Predicted levels of total *V. parahaemolyticus* in oysters after consumer storage in the short supply chain in summer (left) and winter (right).

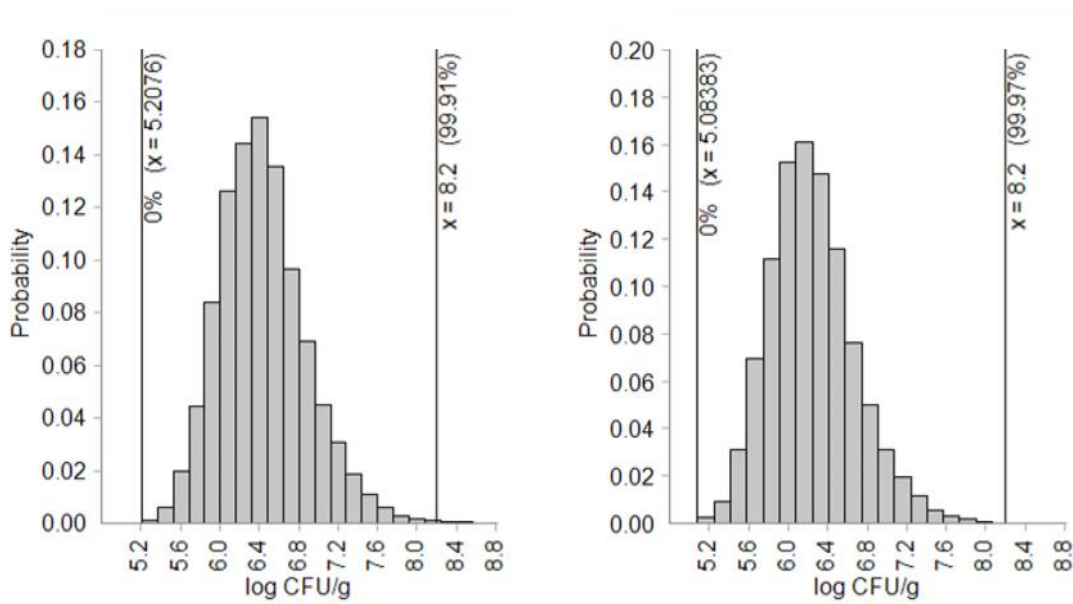
Total viable bacteria count after consumer storage. The mean TVC levels predicted in oysters at the end of the long supply chain were slightly higher for the summer ( $8.4 \log_{10}$  CFU/g) than for the winter ( $7.9 \log_{10}$  CFU/g) scenario. In general, levels of TVC were above  $6.1 \log_{10}$  CFU/g in summer and  $5.9 \log_{10}$  CFU/g in winter (Figure 25). When a reference concentration of  $8.2 \log_{10}$  CFU/g was set, a total of 43.25% and 58.84% of the oysters were below that level in summer and winter, respectively.



**Figure 25.** Predicted total viable bacteria counts levels in oysters after consumer storage in the long supply chain in summer (left) and winter (right).

The mean TVC levels predicted in the short supply chain at the time of consumption were similar for the winter ( $6.2 \log_{10}$  CFU/g) and for the summer ( $6.4 \log_{10}$  CFU/g) scenario. All oysters were predicted to have levels above 5.2 and  $5.1 \log_{10}$  CFU/g for summer and winter seasons, respectively (Figure 26). The percentage of oysters below the reference TVC concentration of  $8.2 \log_{10}$  CFU/g was similar for both seasons; a total of 99.91% in summer and 99.97% in winter were observed.





**Figure 26.** Predicted total viable bacteria counts levels in oysters after consumer storage in the short supply chain in summer (left) and winter (right).

**Sensitivity analysis.** The influence of input factors on selected model outputs was analysed graphically using tornado (Appendix B1) and spider plots.

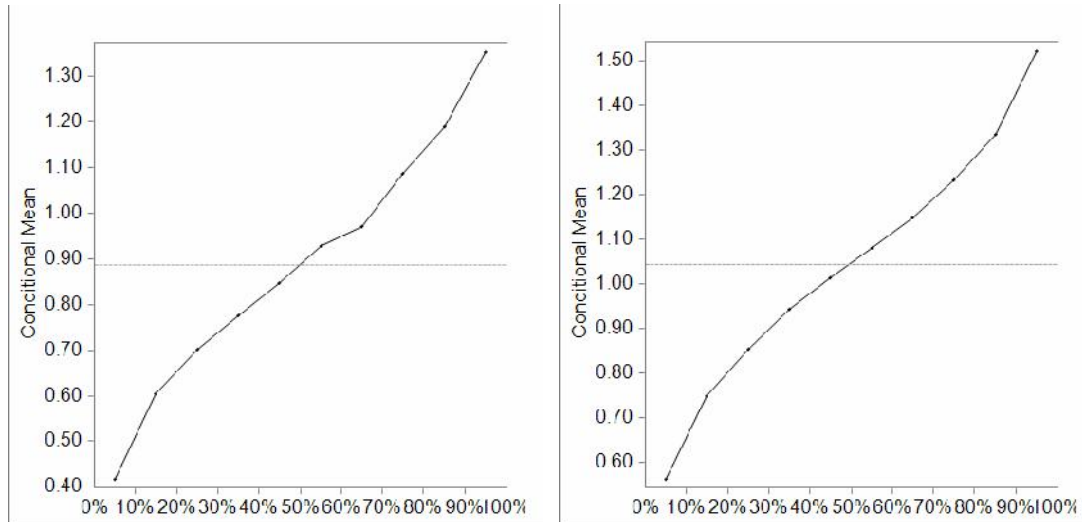
*V. parahaemolyticus* after consumer storage. The most influential input factor for *V. parahaemolyticus* after consumer storage for the two different supply chain scenarios and the two seasons studied was SST (Table 19). The spider plots for the summer scenarios showed that sampling the highest SST values from its distribution would increase the predicted mean *V. parahaemolyticus* levels to 1.4 and 1.5 log<sub>10</sub> CFU/g (Figure 27) for the long and short supply chains, respectively. The opposite effect was observed

when sampling the lowest SST values; the predicted mean *V. parahaemolyticus* levels would decrease to 0.4 and 0.6 log<sub>10</sub> CFU/g for the long and short supply chains, respectively.

**Table 19.** Summary rank (higher:1 to lower:5) for tornado plots analyses of model inputs for *V. parahaemolyticus* levels at consumer storage.

Rank	Long supply chain		Short supply chain	
	Summer	Winter	Summer	Winter
1	SST (harvest)	SST (harvest)	SST (harvest)	SST (harvest)
2	T (transfer)	t (storage at depot)	T (preparation)	T (storage at retail)
3	t (storage at depot)	T (storage at retail)	T (storage at retail)	T (storage at consumer)
4	T (preparation)	T (storage at consumer)	T (transfer)	t (storage at consumer)
5	T (consumer transport)	t (storage at consumer)	T (storage at consumer)	t (storage at retail)

\* SST: seawater surface temperature, T: temperature, t:time.



Grey line showing predicted mean value in log<sub>10</sub> CFU/g. X-axis: cumulative percentiles.

**Figure 27.** Spider plot showing the influence of seawater temperature in the predicted mean levels of *V. parahaemolyticus* after consumer storage for the long (left) and the short (right) supply chain in summer.

Total viable bacteria count after consumer storage: The importance of the inputs was similar for summer and winter but differed between supply chain scenarios (Table 20). Time of storage at depot followed by temperature of storage at retail were the most influential input factors for the long supply chain independently of the season. However, TVC levels at consumption for the short supply chain scenario were more influenced by temperature of storage at retail followed by temperature of storage at consumer.

**Table 20.** Summary rank (higher:1 to lower:5) for tornado plots analyses of model inputs for total viable bacteria count levels at consumer storage

Rank	Long supply chain		Short supply chain	
	Summer	Winter	Summer	Winter
1	t (storage at depot)	t (storage at depot)	T (storage at retail)	T (storage at retail)
2	T (storage at retail)	T (storage at retail)	T (storage at consumer)	T (storage at consumer)
3	T (transfer)	T (storage at farm)	t (storage at retail)	t (storage at retail)
4	T (storage at consumer)	T (storage at consumer)	T (preparation)	T (preparation)
5	T (storage at depot)	T (storage at depot)	T (transport at retail)	t (storage at consumer)

\* SST: seawater surface temperature, T: temperature, t:time.

## 4.4 Discussion

Cold chain management in the oyster industry is necessary to ensure quality and safety throughout the supply chain. However, temperature control can be complicated and oysters experiencing temperatures above the recommended 10°C during supply chain have been observed in Australia (8, 132). A stochastic model to evaluate *V. parahaemolyticus* and TVC levels in oysters during distribution (from farm to consumer) can help to analyse the

effects of temperature on microbial growth during supply chain taking into account the uncertainty and variability present.

In this case study, the changes in *V. parahaemolyticus* and TVC concentrations in oysters were predicted for two different oyster supply chain lengths (the simulation predicted an average duration of 2.5 versus 6.0 d) in winter and summer. The output data were expressed in probabilities which could be used to quantify the percentage of oysters with different bacterial levels.

In general, the predicted levels of *V. parahaemolyticus* in oysters after consumer storage were higher in summer than winter. This was expected as initial levels of the bacteria in oysters are higher in summer than in winter, as observed in previous studies (63, 176). Moreover, bacterial growth is expected to be higher at higher ambient temperatures. The predicted mean levels of *V. parahaemolyticus* after consumer storage were similar for the long and the short supply chains in summer. However, the percentage of oysters predicted to contain *V. parahaemolyticus* >1 CFU/g was higher for the short than the long supply chain. In winter, the maximum levels of *V. parahaemolyticus* after consumer storage were lower for the long than the short supply chains. These observations showed that in this particular simulation the conditions in the supply chain were not only preventing *V. parahaemolyticus* growth but cause reduction in their numbers so that the percentage of oysters containing the bacteria is reduced during transport and storage which helps to reduce consumers exposure to oysters containing high levels of *V. parahaemolyticus*.

The model was also used to predict changes in TVC as an indicator of oyster shelf-life. In contrast to the predicted changes in *V. parahaemolyticus*, supply chain length had more

influence on TVC levels at consumption than harvest season. For example, the percentage of oysters above the reference level of  $8.2 \log_{10}$  TVC in the long supply chain was 56.75% while in the short supply chain it was only 0.09% in summer. The same was observed for winter, with 41.16% of the oysters exceeding the reference level in the long supply chain while only 0.03% of the oysters were predicted to exceed that level in the short supply chain.

The fact that TVC levels were able to increase to unacceptable levels during oyster supply shows that shelf-life management is also necessary for a good cold chain management.

This highlights the importance of comparing TVC (quality) as well as *V. parahaemolyticus* (safety) during supply chain, as they are not necessarily positively correlated. It can happen either that unsafe oysters have good quality, or that safe oysters are of poor overall microbiological quality.

The sensitivity analysis identified variables in the supply chain model that had the greatest influence on *V. parahaemolyticus* and TVC levels at consumption. SST at harvest had the greatest effect on increased *V. parahaemolyticus* levels in oysters. It was observed that selection of some of the highest values in the SST distribution could increase the predicted mean levels of *V. parahaemolyticus* by approximately  $0.5 \log_{10}$  CFU/g in summer for the long and short supply chain. While selection of some of the lowest values in the SST distribution would decrease the predicted mean levels of *V. parahaemolyticus* by approximately  $0.5 \log_{10}$  CFU/g in summer for both supply chain scenarios.

As shown in this case study, the use of tornado plots for sensitivity analysis is very useful as a quick overview to identify the most influential model input parameters (223, 224).

More detailed information was extracted from the spider plots which could identify strategies to reduce the model's output and thereby could be applied to the oyster supply chain to aid management decision of cases where levels are unacceptability high.

The model included real temperature and time data from previous commercial shipments by Tasmanian growers from Madigan (132). However, information for temperatures in the different operations was available only for one supply chain in summer and two in winter. In general, missing data for transport times were obtained from Google Maps and temperature data from the BOM. Some assumptions included in the model are:

- *V. parahaemolyticus* levels at harvest were estimated from SST information using a model which was developed in the USA. The extent in which *V. parahaemolyticus* grow at different SST in Australia may show a different relationship.
- Storage temperatures on farm and at depot for winter were approximately the ASQAP recommendation (10°C).
- Storage times at retail and during consumer storage were both assumed to be approximately 12-36 h.
- Storage temperature at retail was based on recent information available for the USA oyster studies. It could be a different situation in Australia.
- Storage temperature at the consumer level was based on a recent survey in refrigerator temperature in New South Wales. Refrigerator conditions in other states may be different.

- Consumer transport times were based on a survey in Melbourne. Different times may be observed for other states.

The case presented was limited to shipments from Tasmania for simplification of the study. However, differences in supply chain practices have been observed for other states in Australia. In New South Wales, the proportion of producers with access to chillers and refrigerated transport is much lower, but transport chains are much shorter compared with other states (132).

Another important factor that needs to be considered when interpreting the results in this case study is measurement of ambient air temperature. The time for oysters to reach the ambient temperature will not be immediate. In fact, a detailed assessment of a PO supply chain in Australia indicated that it could take up to 60 h for product temperature to cool to 10°C. Another important consideration is that temperature will vary depending on the configuration of the pallet: hollow-style pallets appeared to be more efficient in cooling in comparison to solid-style. The location of the temperature loggers will also have an effect. For example, differences in temperatures inside a truck because of the lack of capacity to remove heat from the load is expected to happen in Australia (132).

An estimation of TVC is used as an index in many seafood standards (107). Microbial criteria for satisfactory oysters at the wholesale level have been set at 5.7 - 6.2 log<sub>10</sub> CFU/g by the USFDA (15, 21). However, a higher level of 10<sup>7</sup> log<sub>10</sub> CFU/g was found to correlate with the maximum shelf-life for purified PO stored at cold temperatures (0 - 10°C) (43).

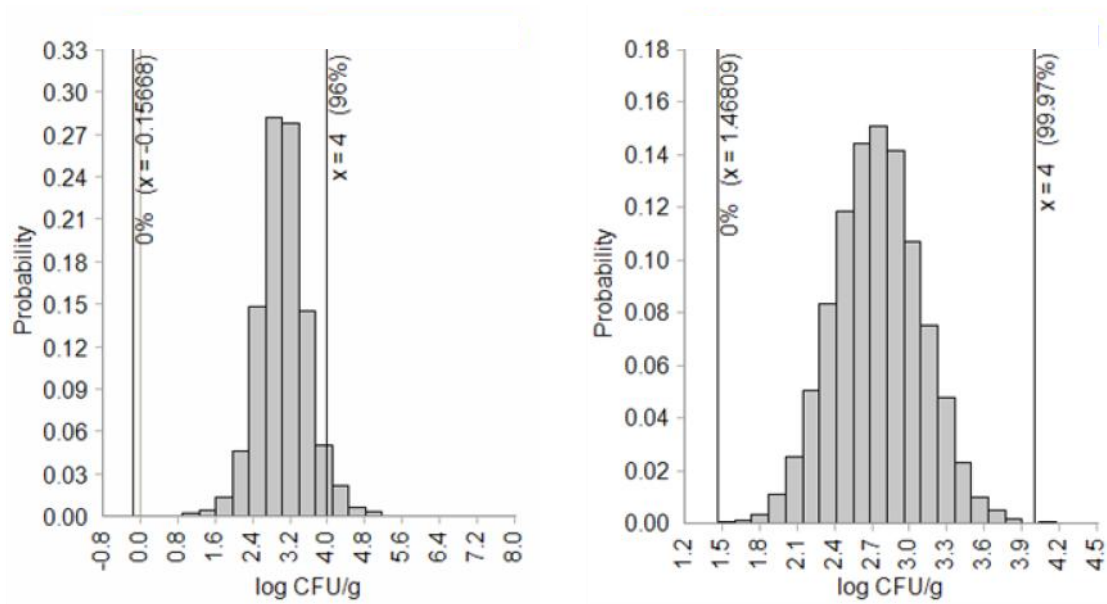
This level was also used as criteria for acceptable quality in shelf-life extension studies (42,

118). In this case study, a higher TVC level of  $8.2 \log_{10}$  CFU/g was selected based on the average gapping times observed during kinetic studies in Chapter 2. Differences in TVC levels correlation with shelf-life could be due to the fact that oysters used here were not purified and starting TVC levels were higher.

The average temperatures for transport and storage up to retail for this case study were below 14.5°C. On rare occasions, oysters can reach temperatures as high as 25°C during transport in Australia (132) which could produce a public health treat, as well as loss of product.

To assess the importance of a temperature abuse event, a what-if scenario was executed with the stochastic model in which all transport steps up to retail and storage at retail were set to a constant value of 20°C. The simulation predicted 2.6 d for the short and 5.7 d for the long supply chain. The predictions showed that the increase in temperature would translate to an increase of *V. parahaemolyticus* after consumer storage to higher levels to the 10,000 MPN/g criterion recommended by the USFDA (22). The percentage of oysters accumulating higher levels than the recommended would be 0.03 and 4% for the short and the long supply chain, respectively, in summer (Figure 28). Apart from the increase in risk for human disease, the probabilities of a quality decrease and thereby a loss in product could be expected. In fact, the percentage of oysters predicted to contain TVC levels above the  $8.2 \log_{10}$  CFU/g reference were 100% for the long supply chain and 71.7% for the short supply chain in summer.





**Figure 28.** Temperature abuse what-if scenario: predicted levels of total *V. parahaemolyticus* in oysters prior to consumption in summer for the long (left) and the short (right) supply chains.

Knowledge of the supply chain temperature profile can also be important in cases where temperature is too low. It has been noted that cold stress can result in oyster mortality (1). Industry has reported that cold abuse often occurs when the insides of trailers are baffled to transport different types of products at different temperatures and oysters are placed in the bay next to frozen products. Cold abuse has resulted in significant losses of product being transported in Australia, particularly in the long complex chains such as South Australia to far north Queensland (132).

The application of predictive microbiology in stochastic approaches for evaluation of the supply chain has several benefits. It allows calculating the percentage of product that will be accepted at the end of the supply chain regarding safety as well as quality. The model can be used to control performance objectives in order to meet food safety objectives which can be used to meet public health goals such as an appropriate level of protection (ALOP) (201). Another advantage is the flexibility in data analysis which can be used as an educational tool to demonstrate the influence of temperature. For example, it can show the effect in bacterial growth for temperature profiles which do not follow the relevant standard. It can also be used to observe the possibility of applying short distance supply chains during winter without refrigeration which could help to design supply chain length. This example of the use of such tools can help industry and regulators to optimize time temperature regimes that assure safety and quality while providing operational flexibility.

Risk managers use QMRA as a science-based tool for making decisions regarding health risk. This case study is an example of a stochastic approach for oyster supply chains which can be considered for exposure assessment. However, this case study ends at the moment the consumer takes the product from the refrigerator and it does not show the impact of the hazard on the final consumer risk (illness) which will require information about dose-response and servings.

Traceability systems which can monitor the supply chain in real-time will take food safety management to a new level of precision and flexibility (152). Wireless traceability technology has been shown to be a useful tool for real-time microbiological monitoring for the distribution of meat in Australia and fresh fish in Denmark (79, 151). It is suggested that the integration of the presented model in a traceability system would provide

additional valuable information. The oyster industry in Australia would benefit from a traceability system that measures time and temperature in real-time, allowing for example software with an output similar to the one in this case study. As a consequence, monitoring and interventions could be made proactively at any point along the supply chain.

The option of using a TTI device, which has the advantage of being less expensive, to measure the time-temperature history in oysters as it has been done in other fish products (170, 214, 218), could be possible if a quality index for oyster shelf-life is identified.

However, the use of TTI presents a variety of disadvantages. First of all, the approach would be deterministic instead of stochastic and some products will be shown to be spoiled while they are still in good quality. Another important aspect is not having real-time knowledge which may delay the recall of inadequate products. Also, TTI can not provide the history of the product and thereby the operation in which the quality is decreased can not be identified.

In the future, similar probabilistic models could be combined with real-time traceability systems to analyse microbial levels in oyster supply chains. As a result, more precise recommendations and increased flexibility in decision making would improve quality and safety management for the oyster industry. Moreover, this study shows possible advantages of using predictive and stochastic modelling for reduction of uncertainty and variability during the oyster supply chain for exposure assessment in risk assessment.

## **5 - Influence of storage temperature on bacterial communities in live Pacific oysters (*Crassostrea gigas*)**

### **5.1 Introduction**

The oyster industry is required to satisfy consumer demands for high quality and safe products that at the same time have the longest shelf-life possible.

The quality of oysters differs to that of other seafood products due to its chemical composition as well as its ability to survive out of water for several weeks (1, 203). After harvest, oysters shells close, trapping water and associated microflora, along with a consequent decrease in oxygen and increase in waste accumulation (49). Changes in the microbial composition of oysters during the post harvest period and the rate of decomposition will be influenced by the initial types and number of micro-organisms present as well as the storage and handling conditions. Consequently, knowledge of the bacterial changes in stored oysters will help identify the organisms involved in spoilage and the best storage conditions to optimize oyster shelf-life.

As culturing techniques are currently limited for most bacteria, microbial ecologists use molecular taxonomic techniques based on the universal marker gene encoding for the 16S rRNA (36, 181, 198). Among different culture-independent techniques, Terminal Restriction Fragment Polymorphism (TRFLP) analysis has been proven to be a robust, high-resolution, high-throughput, rapid and cost-effective method for studying the overall view of microbial communities structures (127, 187). However, TRFLP is basically a

fingerprint method and generates only limited information. In contrast, sequencing of 16S rRNA from clone libraries provides phylotype identification and the community composition in the sample can be assessed (33, 204).

In order to prevent a decrease in microbiological safety and quality, post-harvest handling of oysters is controlled by legislation. The ASQAP requires PO to be stored  $\leq 10^{\circ}\text{C}$  after 24 h of harvest (4). However, refrigeration is usually difficult to achieve along the entire oyster supply chain and oysters can be exposed to higher temperatures for short periods of time. In fact, temperatures  $>10^{\circ}\text{C}$  have been observed in surveys during oyster distribution in the USA and in Australia (62, 132). Thereby, it is important to understand how variation in refrigerated and non-refrigerated storage of oysters influences microbial communities that can influence quality and safety. In this regard, there are few reports that describe the bacterial diversity in oysters using molecular methods (92, 99), however changes in bacterial diversity in oysters stored at different temperatures have not been studied extensively.

The aim of this study was to describe changes in bacterial communities of PO over a range of storage temperatures using both TRFLP and clone library analyses. This investigation will help identify bacteria that may be used as potential spoilage indicators, as well as indicators of oyster storage temperature. In addition, knowledge of bacterial diversity may assist in understanding species of bacteria that may be potential competitive flora to control the growth of pathogenic bacteria in oysters.

## 5.2 Materials and Methods

**Oyster samples preparation.** Five of the ten batches of live PO described in Chapter 2 (Table 7) were used for this study. The batches corresponded to oyster stored at  $3.6 \pm 0.1$ ,  $6.2 \pm 0.1$ ,  $14.9 \pm 0.1$ ,  $20.0 \pm 0.1$  and  $30.4 \pm 0.3^\circ\text{C}$ . Samples of oyster homogenates prepared during kinetics studies (Chapter 2, section 2.2) were stored at  $-20^\circ\text{C}$  until molecular analyses were investigated.

**Total viable bacteria count enumeration.** Information about TVC changes for each of the five different oyster storage conditions were obtained from the kinetics studies in Chapter 2.

**Sampling design.** Oyster samples were coded by letters indicating that they were processed after arrival to the laboratory (fresh, F) or after TVC levels were close to the MPD but before gapping was observed (stored, S), and a number for temperature of storage: 4 (3.6); 6 (6.2); 15 (14.9); 20 (20.0) and 30 (30.4°C).

All oyster samples were analysed by TRFLP. Samples tested by clone library included fresh and oysters stored at 3.6, 14.9 and 30.4°C (F4, F15, F30, S4, S15 and S30), as these samples were found to have different profiles based on TRFLP analysis.

Certain clones representing the major proportion of species in the clone libraries were grown on agar plates and tested by TRFLP to determine fragment sizes. The TRFLP

profiles from clones were then used to identify fragments appearing in TRFLP tests of oyster homogenates.

**DNA extraction.** Oyster homogenates (200 µl) were thawed at room temperature and DNA extracted using the FastDNA<sup>®</sup> Spin Kit for Soil (116560200, MP Biomedicals, Australia) according to the manufacturer's instructions.

**Terminal restriction fragment length polymorphism analysis.** TRFLP analysis was performed as indicated by Powell et al. (181) with some modification. Oyster homogenate DNA was extracted from triplicate subsamples and the 16S rRNA gene from a 2 µl isolated DNA amplified using a 30 µl reaction containing 15 µl ImmoMix<sup>™</sup> (Bioline, NSW, Australia) and 0.07 µM of primers 10F (D3-GAGTTTGATCCTGGCTCAG-3') and 907R (D4-CCGTCAATTCCTTTGAGTTT-3'). 10F and 907R primers were 5' end-labelled with WellRED dye D3 and D4 (SigmaProligo, New South Wales, Australia), respectively. The thermal cycling program consisted of 10 min initial denaturation step at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, with a final elongation step of 7 min at 72°C. PCR reactions were conducted in duplicate and then mixed to avoid bias; 52 µl of the total PCR product was purified using the UltraClean<sup>®</sup> PCR Clean-Up Kit (MB12500, Geneworks, SA, Australia) with DNA eluted using a volume of 60 µl. PCR amplification products were visualized and assayed using agarose gel electrophoresis.

The purified fluorescently labelled PCR products were then digested with 5U of the restriction enzymes *HaeIII*, *Rsa I* or *Alu I* (New England Biolabs, Queensland, Australia). For each enzyme, a 15 µl sample from the purified PCR product was digested for 3 h at 37°C using a thermocycler. The digests were diluted to a 5-fold and 10 µl of each digest was purified by ethanol precipitation within a 96-well plate. The purified digests were resuspended in 30 µl of CEQ sample loading solution (PN608082, Beckman Coulter, Inc., CA, USA) with 0.25 µl of GenomeLab size standard kit 600 (PN608095, Beckman Coulter, Inc., CA, USA). All samples were prepared in the same 96-well plate and examined at the same time. The fragments were separated on a Beckman Coulter CEQ Genetic Analysis system (Beckman Coulter, Inc., CA, USA). The CEQ<sup>TM</sup> 8000 software (Beckman Coulter, Inc., CA, USA) was used to obtain a fragment list with information regarding fragment size measured in base pairs and fragment peak area measured in Relative Fluorescent Units (RFU) for each digest sample.

Normalization procedures were applied prior to statistical analysis. Profiles for each set of sample triplicates were edited by eliminating non-reproducible fragment peaks and averaging shared fragment peaks. Some fragments with a fragment peak area <300 RFU were binned when not present in all triplicate sample profiles to improve reproducibility. The percentage area was calculated for each fragment, and fragments that made up less than 1% of the total area for a sample were not considered. The data for each dye and enzyme were aligned using the T-Align software (<http://inismor.ucd.ie/~talign/> [accessed 2/05/11]) and the data for the three enzymes and the two dyes were combined into one matrix of percentage fragment area for fragment length for all samples.



The Primer6 package (Primer-E Ltd) was used to analyse the data in order to observe differences in the microbial communities. Data were converted to a matrix of Bray-Curtis coefficients. Non-metric Multidimensional Scaling (MDS) plots were used to explore relationships between groups of samples based on the strength of the similarities and dissimilarities. The result of the MDS ordination is a map where the position of each sample is determined by its distance from all other points in the analysis. In a MDS plot the term “stress” is a measure of goodness-of-fit of the final plot. A stress value greater than 0.2 indicates that the plot is close to random and a value lower than 0.2 indicates a useful two-dimensional picture.

Analysis of Similarity (ANOSIM) procedure was used to examine statistical significance between samples. ANOSIM produces a test statistic (R) which ranges from -1 to 1. Objects that were more dissimilar between groups than within groups were indicated by an R statistic approaching 1. An R value of 0 indicated the null hypothesis was true. A level of significance (*p*-value) was produced from the analysis by permutation analysis (*n*=999). For this study, a *p*-value <0.05 was considered significant.

**Clone library construction and analysis.** Clone libraries were constructed for the six oyster homogenates. Oyster homogenate DNA was extracted from triplicate subsamples and 2 µl used for 16S rRNA gene amplification. PCR reactions of 20 µl included 10 µl ImmoMix™ (BIO-25020, Bioline, NSW, Australia) and 0.15 µM of primers 10F (GAGTTTGATCCTGGCTCAG-3') and 907R (CCGTCAATTCCTTTGAGTTT-3'), commercially synthesized (GeneWorks, SA, Australia). The thermal cycling program

consisted of 10 min initial denaturation step at 95°C, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C, with a final elongation step of 7 min at 72°C.

A sample of 5 µl of each amplified PCR product was mixed and the combined 15 µl of combined PCR product for sample was purified using the UltraClean® PCR Clean-Up Kit (MB12500, Geneworks, SA, Australia) with amplicons eluted into 20 µl water. PCR products were assessed using agarose electrophoresis and then cloned using the TOPO® TA Cloning® Kit for Sequencing (K4575-01, Invitrogen, VIC, Australia) according to the manufacturer's protocol. Clones were subcultured and placed directly into a PCR reaction with vector M13 primers (F: GTAAAACGACGGCCAG and R: CAGGAAACAGCTATGAC). Successful amplifications were precipitated using ethanol to remove unincorporated primers. The 96 purified, dry PCR products for each sample were shipped to Macrogen Inc (Seoul, Korea) for sequencing.

The sequences for all six libraries clones were analysed using BioEdit software (95) with manually edited sequences to remove vector regions, and aligned using the CLUSTAL W (216). Sequences were compared to the GenBank database. Phylogenetic trees were constructed using the Kimura 2-parameter model and the neighbour-joining distance method with 1,000 bootstrap replicates using MEGA v. 5 (212). The 16S rRNA gene sequences from *Thermotoga maritima* and *Coprothermobacter platensis* were used as out-group references for the phylogenetic tree.

Fast UniFrac was used to compare similarity among clone libraries (96). The cluster analysis was used to perform a hierarchical clustering analysis, which is based on distance matrix data applied to Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

The significance difference between communities was done by the paired P-test (parsimony based phylogenetic test). UniFrac tests were performed using 1,000 permutations and calculated with the Fast UniFrac web application (<http://bmf2.colorado.edu/fastunifrac/> [accessed 2/05/11]). Coverage of microbial communities represented by clone libraries was calculated according to Good (86).

To test when clones were chimeric, they were analysed performing BLAST searches on different parts of the gene sequence. Potential chimerical sequences were removed. The 16S rRNA gene sequences generated in this study were deposited in the GenBank database under accession numbers JF827355 to JF827597.

### 5.3 Results

**Conditions at oyster harvest.** Five batches of oysters were collected during the period of October 2008 to December 2008. The mean SST recorded during oyster harvest was 17.1°C, with a minimum of 15.0 and a maximum of 19.4°C. Salinity, dissolved oxygen and pH range were 3.4 to 3.6‰, 11.0 to 11.6 mg/l, and 8.3 to 8.6, respectively.

**Bacterial total viable bacteria count.** Initial TVC counts for the five different fresh oyster batches (F) ranged from 4.49 to 5.30 log<sub>10</sub> CFU/g (Table 21). The levels for TVC for oysters after storage (S) were in the range of 7.18 to 8.02 log<sub>10</sub> CFU/g, and the mean was 7.7 log<sub>10</sub> CFU/g.

**Table 21.** Total viable bacteria count in different Pacific oyster batches depending on storage temperature

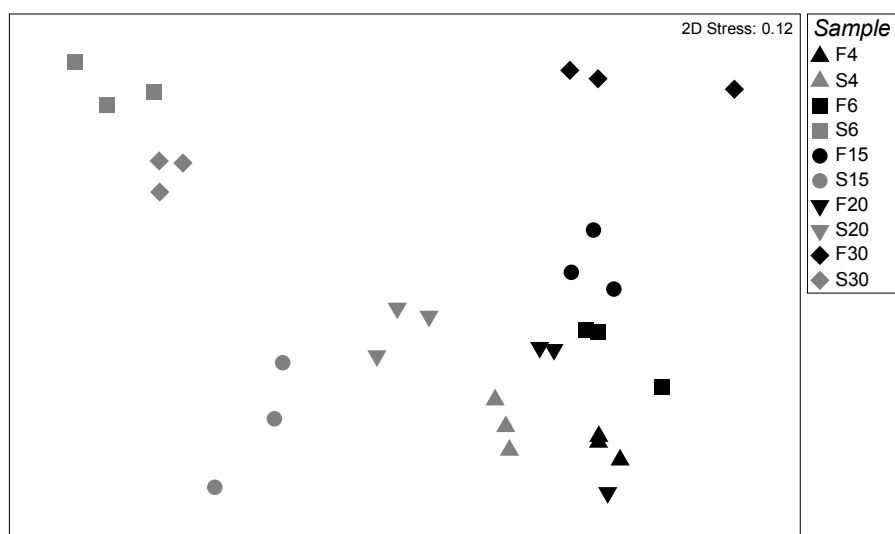
Sample code		F4				S4			
3.6°C	Time (h)	0 <sup>t,c</sup>	25.0	76.5	121.3	191.5 <sup>t,c</sup>	289.0	362.0	432.8 <sup>g</sup>
	log <sub>10</sub> (CFU/g)	5.22	5.44	6.68	7.23	7.81	8.53	8.28	8.66
Sample code		F6				S6			
6.2°C	Time (h)	0 <sup>t</sup>	22.0	75.0	122.0	195.5 <sup>t</sup>	238.3	286.3 <sup>g</sup>	
	log <sub>10</sub> (CFU/g)	5.16	5.16	6.45	7.76	7.98	8.00	8.42	
Sample code		F15				S15			
14.9°C	Time (h)	0 <sup>t,c</sup>	22.8	48.0	71.8	102.2 <sup>t,c</sup>	123.8 <sup>g</sup>	143.8	167.0
	log <sub>10</sub> (CFU/g)	5.30	6.58	7.48	7.62	8.02	8.49	8.30	8.37
Sample code		F20				S20			
20.0°C	Time (h)	0 <sup>t</sup>	8.3	18.8	31.3	47.5	72 <sup>t</sup>	99.5 <sup>g</sup>	123.0
	log <sub>10</sub> (CFU/g)	4.98	5.71	6.57	6.90	7.29	7.37	7.96	8.42
Sample code		F30				S30			
30.4°C	Time (h)	0 <sup>t,c</sup>	5.0	10.0	16.0	23.0	29.3 <sup>t,c</sup>	36.0	45.5 <sup>g</sup>
	log <sub>10</sub> (CFU/g)	4.49	5.26	5.71	6.72	6.85	7.18	7.11	7.29

t: TRFLP sample, c: clone library sample, g: samples gapping (shelf-life).

Shelf-life was dependent on storage temperature; oysters gapped at 432.8 h at the lowest temperature tested (i.e. 3.6°C) and at 45.5 h at the highest temperature tested (i.e. 30.4°C). The average TVC level for the six batches at shelf-life was  $8.2 \pm 0.6 \log_{10}$  CFU/g. Samples chosen for molecular analysis included storage samples before end of shelf-life to identify growing bacteria before spoilage, versus bacteria that proliferate in a dead or dying oyster.

**Terminal restriction fragment length polymorphism analysis.** The bacterial community structure in oyster samples F and S were examined using TRFLP. The differences in

bacterial communities among all samples are shown depending on storage temperature and time in a MDS plot (Figure 29).



Fresh (F, black) and stored (S, grey) Pacific oysters at 4, 6, 15, 20 and 30°C. Stress of the plot = 0.12.

**Figure 29.** Multidimensional scaling plot based on Bray-Curtis similarities of TRFLP data.

ANOSIM analysis showed that the global difference between all samples was large and statistically significant ( $R = 0.94$ ,  $p = 0.001$ ). An overall comparison among oyster samples F and S showed a significant difference ( $R = 0.496$ ,  $p = 0.001$ ). Further analysis in the effect of storage showed that there was no significant difference when oyster sample S4 was compared to all oyster samples F ( $R = 0.043$ ,  $p = 0.384$ ), while oyster samples S6, S15, S20 and S30 were significantly different.

**Clone library analysis.** Six 16S rRNA libraries were generated, three from fresh oysters (samples F4, F15 and F30) and three from the same batches after storage (samples S4, S15 and S30). A total of 518 sequences were analysed successfully from all six samples, from

which 28 were identified as chloroplast-derived, 43 could only be identified to phylum level, and one could not be identified with any cultured species.

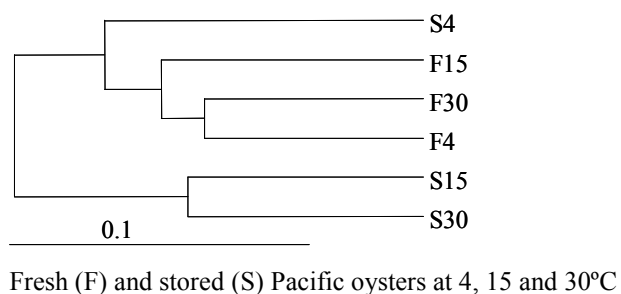
In a global analysis, all clone libraries were found to be significantly different when compared using Fast UniFrac P-test statistical analysis (P-value = 0.001). However, pairwise comparison results presented in Table 22 indicate no differences between all oyster samples F and oysters sample S4, whereas oyster samples S15 and S30 presented significant and highly significant differences, respectively.

**Table 22.** Statistic significance (P-value) of differences between bacterial clone communities calculated based on partial sequences of 16S rRNA

Samples	Significant difference P-value*					
	F15	F30	F4	S15	S30	S4
<b>F15</b>	-	1	0.69	0.015	<0.001	1
<b>F30</b>	1	-	1	0.03	<0.001	1
<b>F4</b>	0.69	1	-	<0.001	<0.001	1
<b>S15</b>	0.015	0.03	<0.001	-	1	1
<b>S30</b>	<0.001	<0.001	<0.001	1	-	0.9
<b>S4</b>	1	1	1	1	0.9	-

\* P-value (parsimony based phylogenetic test, implemented in UniFrac) corrected for multiple comparisons using the Bonferroni correction calculated based on 1000 permutations. <0.001 highly significant, (0.001-0.01) significant, (0.01-0.05) marginally significant, (0.05-0.1) suggestive, >0.1 not significant. Fresh (F) and stored (S) Pacific oysters at 4, 15 and 30°C.

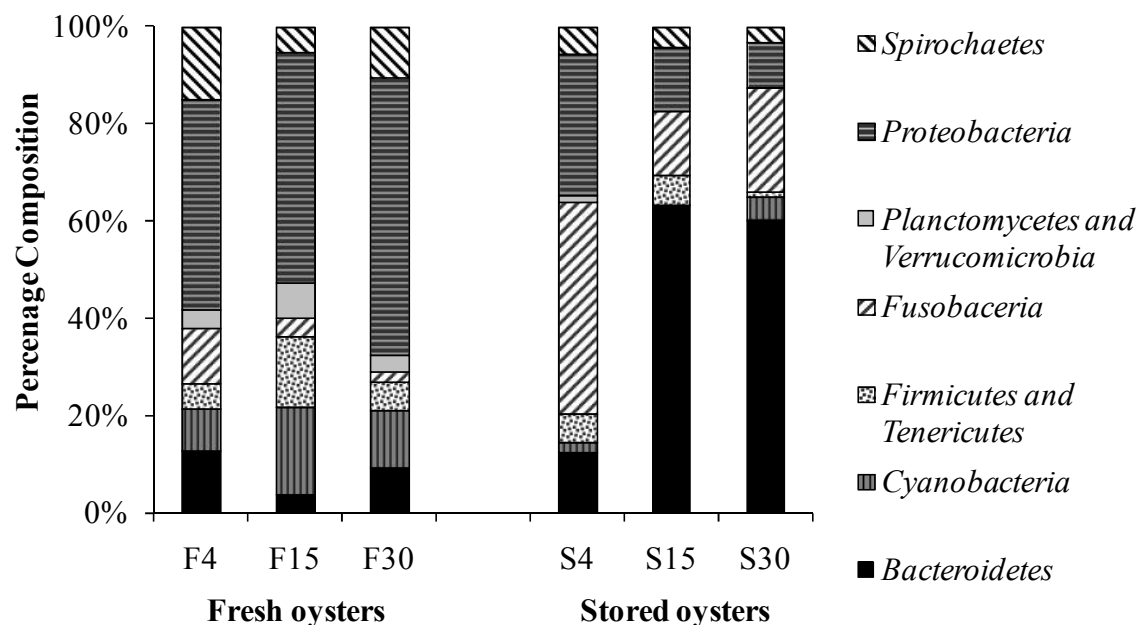
A cluster analysis showed that the libraries for oyster samples F clustered with the library for oyster sample S4 (Figure 30). Clone libraries for oyster samples S15 and S30 formed a cluster distinct from other samples.



**Figure 30.** Cluster analysis of the six different clone library compositions obtained by Fast UniFrac.

The identified taxa for each clone library grouped into nine different phyla (Figure 31). Fresh oyster samples were dominated by *Proteobacteria* making up 43.0 to 57.0% of clones in fresh samples. Bacterial diversity changed after storage and varied depending on holding temperature. Bacterial profiles after storage were more similar for oysters tested at 14.9 and 30.4°C than oyster tested at 3.6°C. Overall, there was a decrease in *Spirochaetes*, *Proteobacteria*, *Planctomycetes* and *Verrucomicrobia*, and *Cyanobacteria*; and an increase in *Fusobacteria*. For oysters stored at 3.6°C, the phylum *Fusobacteria* became dominant (43.8% of clones). In contrast, *Bacteroidetes* made up the majority of clones for oysters stored at 14.9 and 30.4°C, representing 63.0 and 60.2% of the total community, respectively.

## 5 – Storage temperature effects on bacterial communities in *C. gigas*



Fresh (F) and stored (S) Pacific oysters at 4, 15 and 30°C. Bars represent the percentage of clone library composition represented by each phylum or combination of phyla.

**Figure 31.** Clone library composition of bacteria in homogenates of six batches of Pacific oysters.

A total of 447 clones could be readily associated with known bacterial groups. Among all samples, 73 different genera-related were observed (Appendix C1). Clones associated with known bacterial genera typically had 93-99% similarity to described species. Oyster clones which had maximum identity values <93% were referred as related clusters to the closest known validly described species.

For a condensed interpretation of data, only the major clone clusters representing more than 5% of the total composition in each library were discussed here. A total of 17



sequence clusters (Table 23) were compared between clone libraries and represented an average  $72 \pm 8\%$  of the total composition in each library.

**Table 23.** Good's coverage values and representative genera in each clone library for six different oyster samples

Phylum	Class	Closest related genus	Maximum identity (%)	Fresh			Stored		
				F4 Composition (%)	F15 F30	F30	S4 Composition (%)	S15	S30
<i>Spirochaetes</i>									
		<i>Spirochaeta</i>	79-87	9	5	7	1	2	3
		<i>Cristispira</i>	94-95	5	0	3	4	1	0
<i>Proteobacteria</i>									
	<i>Alpha-</i>	<i>Sphingomonas</i>	89-92	8	0	3	3	7	0
	<i>Alpha-</i>	<i>Thalassospira</i>	85-87	9	21	14	9	1	1
	<i>Alpha-</i>	<i>Mesorhizobium</i>	91-97	0	0	21	0	2	0
	<i>Beta-</i>	<i>Polynucleobacter</i>	98	5	4	2	7	0	0
	<i>Epsilon-</i>	<i>Arcobacter</i>	93-99	0	0	0	2	0	6
<i>Fusobacteria</i>									
	<i>Fusobacteriales</i>	<i>Psychrhilyobacter</i>	96-99	11	4	2	44	12	20
<i>Cyanobacteria</i>									
	<i>Chroococcales</i>	<i>Synechococcus</i>	95-99	8	18	10	2	0	5
<i>Bacteroidetes</i>									
	<i>Bacteroidia</i>	<i>Alkaliflexus</i>	86-88	0	0	0	0	38	13
	<i>Flavobacteria</i>	<i>Dokdonia</i>	95-97	0	0	0	1	0	6
	<i>Flavobacteria</i>	<i>Psychroserpens</i>	94	0	0	0	0	1	6
	<i>Flavobacteria</i>	<i>Polaribacter</i>	97-99	0	4	0	3	2	8
	<i>Flavobacteria</i>	<i>Bizionia</i>	96-97	0	0	0	2	4	5
<i>Tenericutes</i>									
	<i>Mollicutes</i>	<i>Mycoplasma</i>	84-88	1	5	1	3	2	0
	<i>Mollicutes</i>	<i>Spiroplasma</i>	81-86	4	5	3	1	4	1
<i>Planctomycetes</i>									
	<i>Planctomycetacia</i>	<i>Rhodopirellula</i>	93-95	0	5	0	0	0	0
<b>Others</b>				40	29	31	16	23	27
<b>Good's coverage (%)</b>				70	90	81	87	92	87

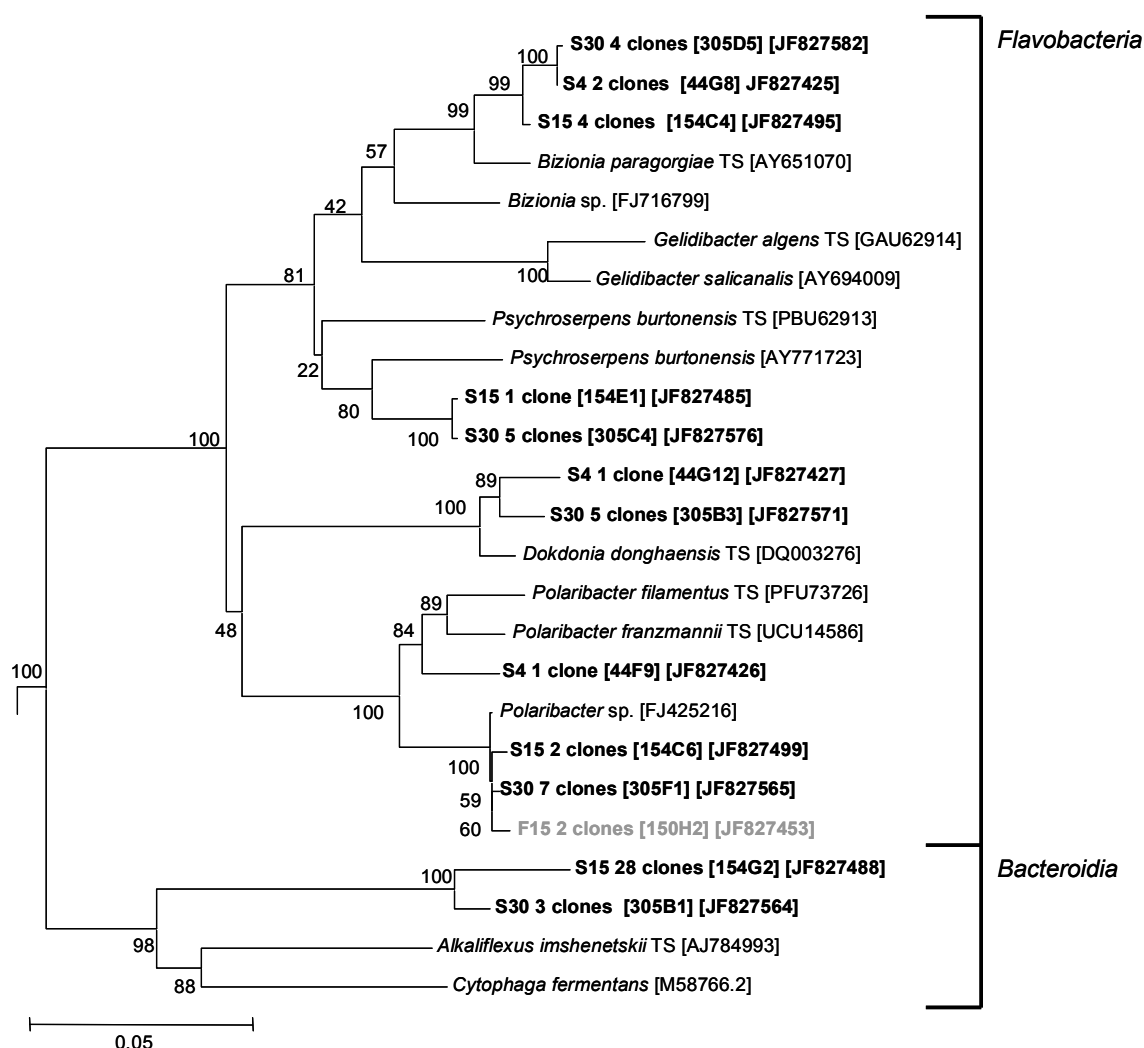
Fresh (F) and stored (S) Pacific oysters at 4, 15 and 30°C.

A decrease in a *Spirochaeta*-related and *Spiroplasma*-related clusters and in *Synechococcus*, and an increase in *Psychrilyobacter* and *Bizionia* spp. were observed in all

stored oysters independent of temperature. Clones belonging to the *Thalassospira*-related cluster were present in all fresh oyster batches (9 to 21%) but decreased to only 1% after storage at 14.9 or 30.4°C. Only one batch of fresh oysters contained high levels (21%) of a clone cluster most closely related to the genus *Mesorhizobium*; however this cluster was not detected after storage. A cluster of clones most closely related to the genus *Alkaliflexus* (Figure 32) was only detected in oysters stored at 14.9 and 30.4°C, representing 38 and 13% of clones, respectively. A cluster of clones grouping within the genus *Polynucleobacter* (Figure 33) was observed in all fresh samples while after storage it was only detected in oysters stored at 3.6°C, constituting 7% of the total composition. A cluster of clones most closely related to the genera *Arcobacter* and *Dokdonia* were not present in fresh oysters but in oysters stored at 3.6 and 30.4°C.

Good's coverage increased in all samples after storage when compared to fresh samples. The lowest coverage of diversity (70%) was found for oyster sample F4 while the highest (92%) was observed for oyster sample S15.

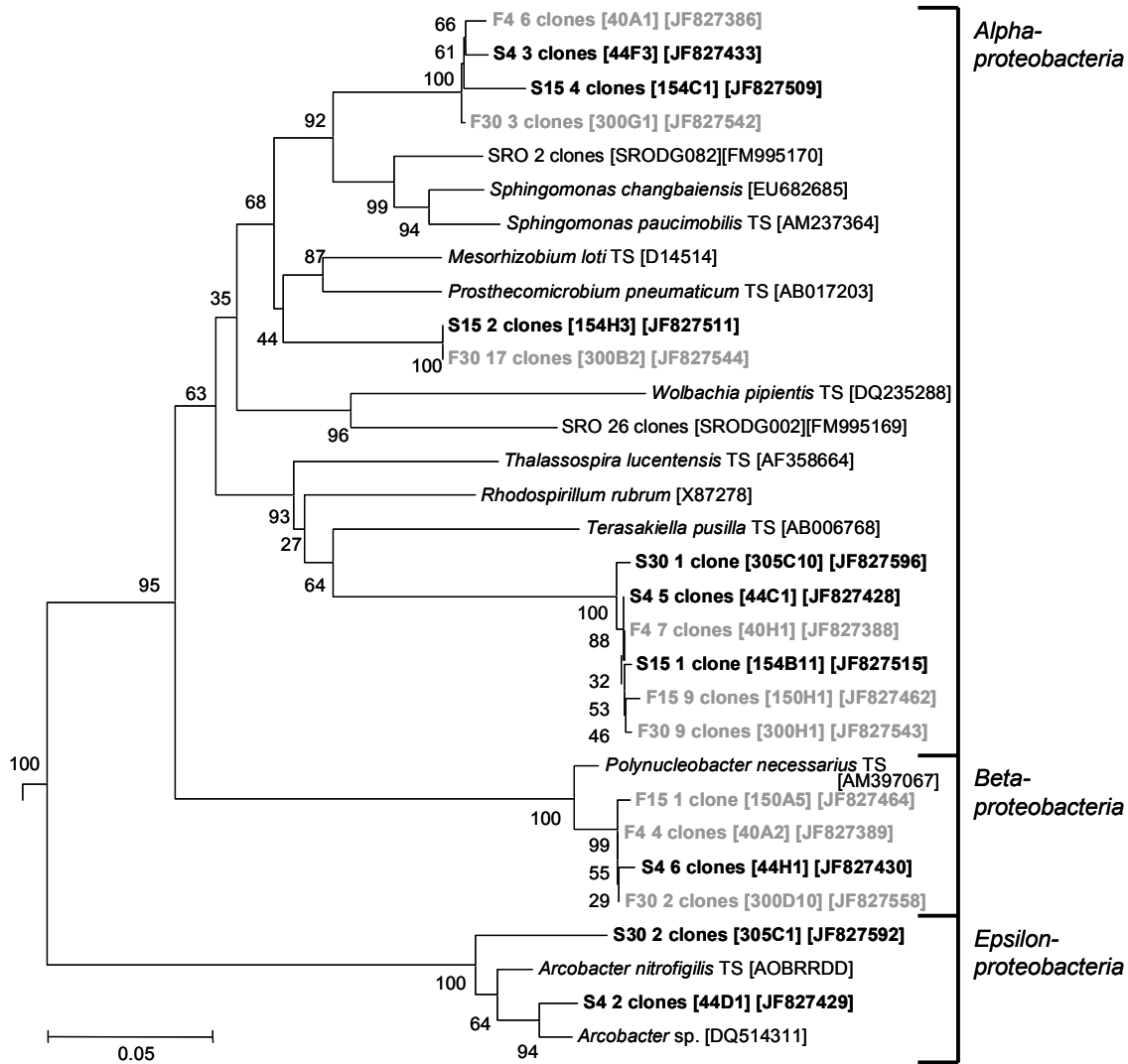
Some of the clones isolated from the digestive gland in SRO in a previous study (92) were included for phylogenetic tree analysis (Figure 33 and 34). The clones did not show any close phylogenetic relationship to clones in PO homogenates.



Samples F4, F15, F30 (grey) and S4, S15, S30 (black). Codes in brackets are clone reference and GenBank accession number. The numbers at the nodes of the tree indicate bootstrap values for each node. Scale bar represents 5% estimated distance.

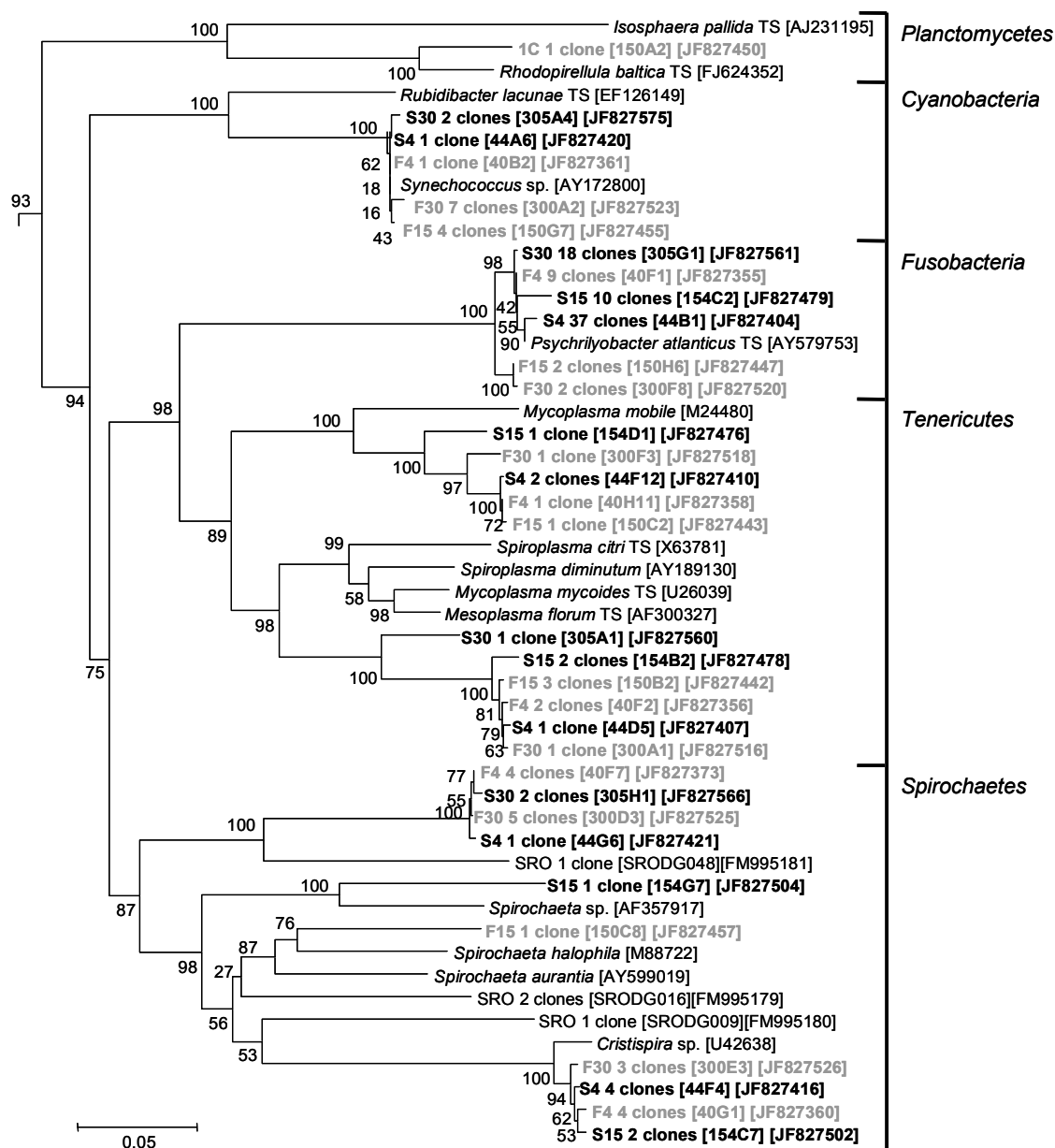
**Figure 32.** 16S rRNA phylogenetic tree of representative genera for the phylum *Bacteroidetes* from sample clone libraries (bold letters) and type strains, based on the Kimura 2-parameter model and the neighbor-joining method.

## 5 – Storage temperature effects on bacterial communities in *C. gigas*



Samples F4, F15, F30 (grey) and S4, S15, S30 (black). Clones identified in published study of Sydney Rock oyster (SRO) digestive gland (92). Codes in brackets are clone reference and GenBank accession number. The numbers at the nodes of the tree indicate bootstrap values for each node. Scale bar represents 5% estimated distance.

**Figure 33.** 16S rRNA phylogenetic tree of representative genera for the phylum *Proteobacteria* from sample clone libraries (bold letters) and type strains, based on the Kimura 2-parameter model and the neighbor-joining method.



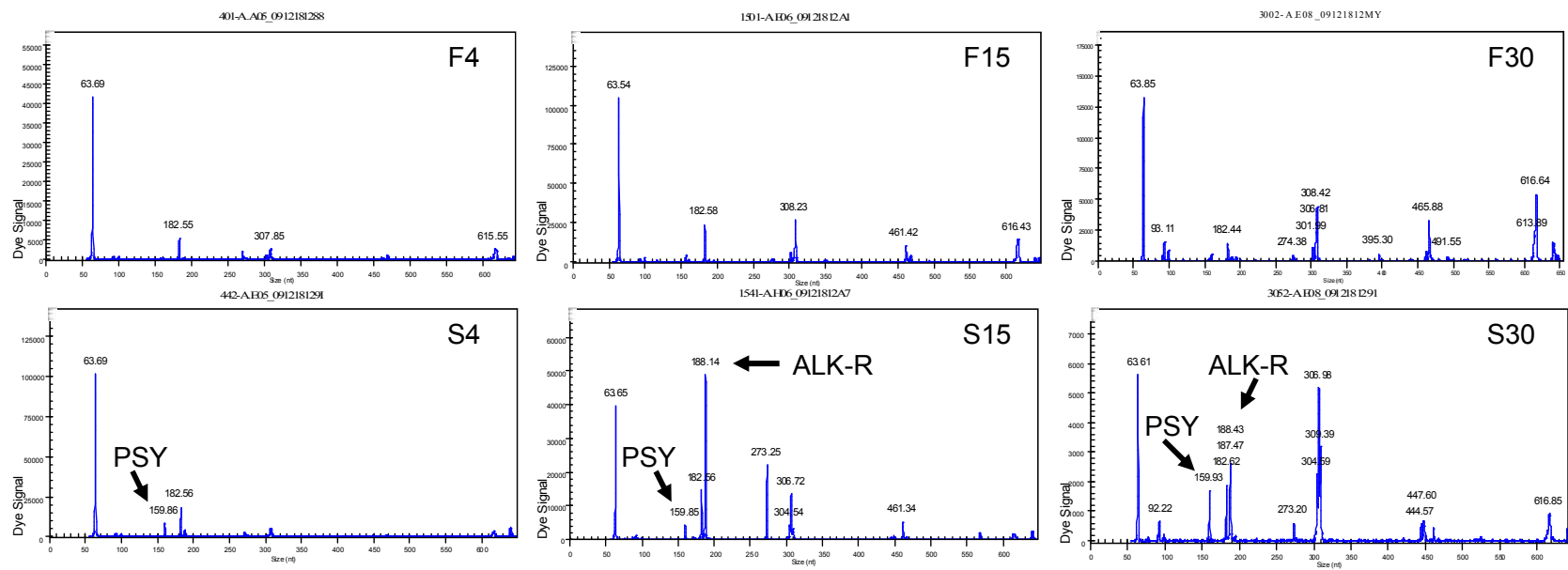
Samples F4, F15, F30 (grey) and S4, S15, S30 (black). Clones identified in published study of Sydney Rock oyster (SRO) digestive gland (92). Codes in brackets are clone reference and GenBank accession number. The numbers at the nodes of the tree indicate bootstrap values for each node. Scale bar represents 5% estimated distance.

**Figure 34.** 16S rRNA phylogenetic tree of representative genera for phyla *Spirochaetes*, *Fusobacteria*, *Cyanobacteria*, *Tenericutes* and *Planctomycetes* from sample clone libraries

(bold letters) and type strains, based on the Kimura 2-parameter model and the neighbor-joining method.

**Terminal restriction fragment length polymorphism analysis in bacterial clones isolated from oyster homogenate during cloning libraries.** After clone library analyses from the oyster homogenate, the TRFLP profiles for clones *Alkaliflexus*-related (JF827488 and JF427564) and *Psychrilyobacter* (JF827404 and JF827561) were performed (Appendix C2).

The TRFLP profiles derived from *AluI* digestion of the four selected clones and different oyster samples were compared. *Psychrilyobacter* spp. had a fragment of 160 bp and appeared only in profiles for all stored oysters. A *Alkaliflexus*-related clone had a fragment of 188 bp and appeared only in oyster samples S15 and S30 (Figure 35).



Fresh (F) and stored Pacific oysters (S) at 4, 15 and 30°C.

**Figure 35.** Terminal restriction fragment length profiles for six different oyster samples. Identification of fragments as *Psychrilyobacter* (PSY) and *Alkaliflexus*-related (ALK-R).

## 5.4 Discussion

The microbiological quality and safety of oysters changes within supply chains. During storage and transport processes, different conditions are created in the oyster that will lead to a change in oyster microflora (49). Although requirements exist (4, 20), temperature increases can occur during transport or storage.

Bacterial communities in oysters have been studied using both traditional culture-dependent (46, 120) and culture-independent (92, 99) methods. However, there is a lack of knowledge about the effect of storage temperature on these communities. Using a combination of two 16S rRNA-based methods, bacterial communities present in fresh and stored oysters were studied.

TRFLP analysis showed that bacterial community composition was significantly different for the five different fresh oyster samples harvested on different days versus the same oyster batches stored at different temperature conditions, based on analysis of similarities (ANOSIM; Global R = 0.496, p = 0.001). This result indicated that differences among storage were significant in respect to the variability of community structure observed in fresh oysters studied here.

The most dominant bacterial groups in the three batches of fresh oysters were similar and dominated by members of class *Alphaproteobacteria*. A very large group which consist of many species isolated from marine environments including hydrocarbon-degrading bacteria (e.g. *Thalassospira* and *Sphingomonas* spp.) (122). A dominant cluster was represented by one related to *Terasakiella* and *Thalassospira*, aerobic bacteria found in



marine environments (128, 200), and thus could represent a novel genus. Twenty-one percent of clones from one batch of fresh oysters formed a cluster adjacent to a soil-associated genus *Mesorhizobium* (109) and aquatic *Prosthecomicrobium* (171) and also appears to represent a novel group at the genus level. *Polynucleobacter* spp., a genus which belongs to the class *Betaproteobacteria*, was present in all fresh samples. These bacteria are usually isolated from freshwater habitats but can also be present in brackish-aquatic environments (94, 220).

The presence of *Proteobacteria* in PO has been previously shown by fluorescent in situ hybridization (99). *Alphaproteobacteria* also formed a major part of the microbiota found in Mediterranean oysters (*Ostrea edulis*) and in SRO (92, 182). One alphaproteobacterial sequence (SRODG002) representing 26 clones from a study of SRO was included in the phylogenetic tree (Figure 33) showing that it belongs to another group of species distinct from the clones of PO identified in this study.

*Arcobacter* spp., which belong to the class *Epsilonproteobacteria* are an abundant and common component in depurated Chilean oysters (*Tiostrea chilensis*) (192). In this study, clones related to *Arcobacter* spp. were also detected in PO after storage at 3.6 and 30.4°C (2 and 5% of the clone composition, respectively) which indicates that this genus is present but not necessarily dominant.

Studies using culture-based methods have usually identified *Gammaproteobacteria* as dominant. In the case of PO, cultured isolates are most commonly *Pseudomonas* and *Vibrio* spp. (46, 120), while in the Tropical oyster (*Crassostrea iredalei*), *Shewanella* and *Vibrio* spp. (157) are more frequently isolated.

Our results indicated that PO actually had high bacterial species diversity with up to 73 different genera-related identified clones among all samples. However, little similarity to culture-dependent studies was observed. Oysters may be a reservoir for novel micro-organisms since a similar high and unrealized diversity was found in SRO (92).

Oysters can accumulate high concentrations of pathogenic organisms because of their filter-feeding activity. The different micro-organism that can cause illness in humans associated with oyster consumption are bacteria which originate from human and animal waste (*Salmonella* spp., *Shigella* spp., *Escherichia coli*), from general environment (*Clostridium botulinum* type A and B, *Listeria monocytogenes*) or those that might be present in natural environment (*Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio cholerae*, *Clostridium botulinum*, *Aeromonas hydrophilia*, *Plesiomona shigelloides*) (89, 166). In this study, all oyster batches were harvested when seawater temperature was  $\geq 15^{\circ}\text{C}$  and the occurrence of *Vibrio* spp. under this condition has been previously observed (111, 176). However, we did not observe any *Vibrio* spp. in any of the six clone libraries. This is in agreement with the low numbers of *Vibrionaceae* identified in SRO using clone library analysis (92) and may be a result of the fact that most abundant bacteria in oysters are not culturable on standard agar media under aerobic conditions (191).

*Shewanella* and *Photobacterium* spp. identified previously in oysters could also pose a health threat through the ingestion of contaminated seafood (157, 189). *Shewanella* and *Photobacterium* spp have been associated with septicemia and necrotizing fasciitis from wound infection respectively (175, 230). Members of these genera were also not observed, which suggests that PO from the harvested area do not support these genera or their levels are below the resolution limits of the clone library analysis.

After storage, the bacterial taxonomic diversity in oysters decreased as shown by Good's coverage analysis and the identified bacterial groups changed. These could represent bacteria that strongly respond to the new conditions created in the oyster during storage including less oxygen, accumulation of waste, and interrupted feeding activity.

As observed by clone library statistical analysis, bacterial communities identified in oysters stored at 3.6°C differed to those at 14.9 and 30.4°C. This result indicates that organisms responsible for spoilage may be different depending on the storage temperature. This is in agreement with observations made for various fish species, in which storage temperature can change the microflora responsible for spoilage (90). For example, spoilage in iced Nile perch (*Lates niloticus*) consists typically of *Pseudomonas* spp. while at ambient temperature the microflora can be dominated by *Aeromonas* spp.(91).

In general, stored oysters exhibited a decrease in *Proteobacteria* independent of the temperature tested and an increase in the genus *Psychrilyobacter*, a member of phylum *Fusobacteria*. This genus is an obligately anaerobic halophile which is able to grow well at low temperatures and it has been recently isolated and described from marine sediments and marine animals (163, 202, 236). Bacteria species related to the genus *Alkaliflexus* also seem to become more abundant after storage at 14.9 and 30.4°C but not at 3.6°C, showing a similar behaviour to some *Alkaliflexus* strains which are not able to grow at cold temperatures (237). *Lactobacillus* and *Pseudomonas* spp. were found to be the major component in PO after storage at cold temperatures (5 - 10°C) (43, 206) while a higher temperature of 18°C, *Pseudoalteromonas* spp were the most abundant bacteria in spoiled Chilean oysters (*Tiostrea chillensis*) (193).

The spoilage of oysters has been assumed to be driven by fermentative organisms because of the glycogen content in oysters (49). We have observed an increase in predominantly halophilic, anaerobic or facultatively anaerobic, fermentative bacteria including *Psychrilyobacter*, *Alkaliflexus*, *Polynucleobacter* and *Polaribacter* after storage (87, 94, 236, 237).

An estimation of TVC is used as an index in many seafood regulatory standards (107). However, in some cases only a fraction of the total flora contribute to the production of off-odours and off-flavours instead of all total numbers (90). In these cases, it is the growth of SSO which induce the major changes and thereby counts of these organisms are better related to shelf-life.

*Shewanella* and *Pseudomonas* spp. are examples of SSO in chilled fish and *Photobacterium* spp. in modified atmosphere stored marine fish (90). We have identified a notable increase in *Psychrilyobacter* spp. after storage independently of the temperature that may be used as a possible indicator organism in spoilage for future shelf-life studies in oysters. Moreover, it was observed that *Polynucleobacter* spp. also only increased in oysters stored at low temperature (3.6°C) and sequences related to the genus *Alkaliflexus* only increased at higher storage temperatures (14.9 and 30.4°C). These species may be useful as indicator organisms for temperature control.

In general, there was good complementation between the two molecular methodologies used. TRFLP analysis of some selected clones from the oyster samples allowed identification of fragment peaks generated in TRFLP studies from oyster homogenate DNA for *Psychrilyobacter* and those related to *Alkaliflexus*. However, we could not

differentiate major fragments and they may be a result of a mixture of different bacteria. Although fragment identification in TRFLP profiles could be further investigated to classify fresh from stored samples, other molecular methods (e.g. PCR) may be more suitable for this purpose.

Previous comparisons show that the richness estimated by TRFLP is lower than that estimated from clone libraries. Some explanations are that TRFLP can miss rare species due to detection limits as some may not generate enough fluorescently labelled PCR amplicon or due to the occurrence of binning in TRFLP, where two different species are counted as one TRFLP fragment because they generate the same size fragment (174). In order to overcome this limitation, we have used three different restriction enzymes, as sequences that bin together with one restriction enzyme might produce different-sized fragments when targeted by a different restriction enzyme.

In this study, oysters were alive during storage and the host-defence system of the organism may have protected it against spoilage. Storage of shucked oysters may lead to rather different patterns in microbial diversity. We took into account storage in open trays that can also differ for oysters stored in sacks since oysters kept tightly sealed are forced to metabolize anaerobically (203).

The different fresh oyster samples analysed in this study were sampled from the same harvest area different days and showed similar bacterial profiles. Bacterial communities in the Eastern oyster (*Crassostrea virginica*) have been observed to differ depending on the local environment conditions (108). It was suggested that differences in productivity or salinity in estuaries could have an influence in the bacterial diversity. Different bacterial

communities in fresh oysters may have an influence in the predominant spoilage organisms detected.

In conclusion, bacterial communities in PO were found to be diverse but microbial diversity shifts considerably between fresh and stored oysters and between oysters stored depending on temperature. In future studies quantitative correlation of the identified species and the freshness of oysters are required in order to confirm that the predominant microbes detected here represent significant spoilage indicators. Further studies could determine if they are antagonistic to human oyster bacteria pathogens.

## 6 – Conclusions and future work

### 6.1 Introduction

The oyster industry is an important economic sector in Australia which strives to provide a high quality and safe product to consumers. Consumption of raw or undercooked oysters containing infectious levels of pathogenic *V. parahaemolyticus* can result in gastroenteritis in healthy individuals and septicaemia in susceptible population. In Australia, pathogenic *V. parahaemolyticus* has been isolated from oysters. However, epidemiological data show that the risk of illness is relatively low.

*Vibrio parahaemolyticus* is naturally occurring in seawater environments and can accumulate in oysters to concentrations greater than those found in the surrounding seawater. Oyster harvest sites are tested microbiologically for total or faecal coliform which do not necessarily correlate with the presence of *V. parahaemolyticus*. Currently, there is no existing control which can ensure that oysters do not contain *V. parahaemolyticus* after harvest apart from microbial testing of the product.

Levels of *V. parahaemolyticus* can increase in oysters at growth-permissive temperatures. In order to control this risk, shellfish quality assurance programs (ASQAP, USNSSP) include time-temperature requirements for handling, storage and transport after harvest. However, there are two different issues that need to be addressed: the improvement of temperature control during supply chain and the application of specific temperature requirements depending on oyster species or geographical location.

A combination of the emergence of *V. parahaemolyticus* infections worldwide, potentially enforceable maximum regulatory limits and the effects of climate change increase the need for *V. parahaemolyticus* management tools. Among the different post-harvest operations (e.g. high pressure processing, irradiation) which can reduce levels of *V. parahaemolyticus* in oysters, at present, refrigeration is the most practical control for producing a more natural product. The application of predictive microbiology in oyster supply chain management can help to reduce and maintain acceptable *V. parahaemolyticus* levels in oysters through education and by objectively evaluating the effect of temperature during different supply chain operations on the exposure level for consumers.

## 6.2 Findings

***Vibrio parahaemolyticus* in *C. gigas* temperature model.** The *V. parahaemolyticus* and TVC models in PO can predict the viability of these bacteria based on the effect of post-harvest ambient air temperature in the range of 3.6 - 30.4°C.

- Levels of *V. parahaemolyticus* in artificially contaminated PO were stable at 14.9°C, increased at  $\geq 18.4^\circ\text{C}$  and decreased at  $\leq 12.6^\circ\text{C}$ . This indicates that *V. parahaemolyticus* should not grow in PO after harvest when stored at temperatures recommended by the ASQAP ( $\leq 10^\circ\text{C}$ ).

- The *V. parahaemolyticus* growth model differs to others reported in literature, demonstrating that differences among geographical locations and oyster species need to be considered during *V. parahaemolyticus* risk management.



- The *V. parahaemolyticus* and TVC models were overly fail-safe for SRO, indicating that temperatures controls for this oyster species should be different to manage *V. parahaemolyticus* risk.
- The kinetic data generated in this study were submitted to the FAO/WHO risk assessment group in response to a 2010 Call for Data. These data will be used to evaluate the validity of models used to help nations manage *Vibrio* spp. risk in foods.

**Evaluation of *V. parahaemolyticus* tertiary model.** The scientific knowledge from the models for *V. parahaemolyticus* and TVC in PO were then translated into an Excel<sup>®</sup> tertiary model for use in the oyster industry as a cold supply chain management tool.

- The overall performance of the software program during simulated oyster supply chain scenarios was found to be “fail-safe”. A mean model overestimation of 2.30 for *V. parahaemolyticus* and 2.40 for TVC growth were measured by the bias factor index.
- The software tool allows experts to easily input time-temperature profiles and thereby predict and interpret *V. parahaemolyticus* and TVC levels.
- The tool was designed to suit the oyster industry but can be easily accessed by other users (e.g. food industry, risk assessors, food microbiologists).

**Cold chain management tool for oysters: a case study.** The evaluated predictive models for *V. parahaemolyticus* and TVC in PO were also integrated in a stochastic approach to

incorporate uncertainty and variability present in the oyster supply chains to provide more accurate estimations of commercial operations.

- The probabilistic models are used to understand prevalence and concentrations of total *V. parahaemolyticus* and TVC occurring (and accumulating) at different nodes in oyster supply chains.
- The output data are expressed in probabilities which can be used to quantify the percentage of oysters with different bacterial levels in exposure assessment for oyster risk management.
- TVC and *V. parahaemolyticus* growth showed a different pattern in the case study, highlighting the importance of comparing both models to assess quality as well as safety in supply chains.
- Different operations within supply chains can be examined to identify potential strategies to achieve food safety and/or quality objectives.
- The stochastic model could be used to simulate what-if scenarios for temperature abuse.

**Storage temperature effects on bacterial communities in *C. gigas*.** The effect of storage temperature on bacterial communities in PO was examined using two different molecular techniques, TRFLP and clone library.

- The most dominant bacterial group in freshly harvested PO was *Alphaproteobacteria*, while studies using culture-based methods have usually identified *Gammaproteobacteria*.
- PO presented high microbial diversity with up to 73 different genera-related identified clones among all samples (fresh and stored). Phylogenetic studies showed new clusters for oyster bacteria that were not closely related to known bacteria, suggesting that oysters may be a reservoir for novel micro-organisms.
- A notable increase in *Psychrilyobacter* spp. after storage was identified independent of the temperature and should be investigated as a possible indicator of spoilage.
- Some bacterial shifts were dependent on storage temperature. *Polynucleobacter* spp. only increased in oysters stored at low temperature (3.6°C) while sequences related to the genus *Alkaliflexus* only increased at higher storage temperatures (14.9 and 30.4°C). These species may be useful as indicator organisms for temperature control.

### 6.3 Future

The integration of the Excel<sup>®</sup> predictive software program and/or the stochastic model in a wireless traceability system would provide valuable information to the oyster industry. The measurement of time and temperature in real-time would allow predictions of levels of

*V. parahaemolyticus* and TVC (deterministic model) or percentage of oysters with different levels of *V. parahaemolyticus* and TVC (stochastic model). As a result, monitoring and interventions could be made proactively at any point along the supply chain. Thereby, risk managers could have more objective recommendations and increased flexibility in decision-making.

The identified bacterial species after oyster storage studies could be used in future projects to find a quantitative correlation with oyster shelf-life to confirm if the predominant microbes detected in this study represent significant spoilage indicators. Additional information about TVC levels necessary for organoleptic rejection of oysters at different temperatures would also help in the interpretation of model outcomes.

Further research could determine if predominant bacterial species in fresh and stored oysters are antagonistic to human bacterial pathogens, thus helping to design new control measures. Future research could include kinetic studies at different temperatures and comparison with *V. parahaemolyticus* viability kinetics.

Differences in bacterial growth were observed between the two oyster species studied. Future work is needed to understand the role of oyster physiology and host defence systems on levels of *V. parahaemolyticus* in SRO.

Determining a correlation between the number of total and pathogenic *V. parahaemolyticus* could be used to extrapolate model predictions to pathogenic strains. However, performing more kinetic studies using pathogenic *V. parahaemolyticus* strains would be necessary to ensure the same relationship is observed over different storage temperatures.

Information about the incidence and levels of pathogenic *V. parahaemolyticus* strains in Australian oysters and the relationship with environmental factors (e.g. SST, turbidity, salinity) could help build an appropriate mathematical model to predict *V. parahaemolyticus* levels after harvest, analogous to the USFDA model for American oysters.

It has been suggested that oysters from different geographical locations may present different bacterial communities and thereby differently influence the growth of *V. parahaemolyticus*. Additional data about bacterial community composition as well as genetic differences in *V. parahaemolyticus* strains will enable the production of models more suitable for growing regions.

The stochastic model was used for a Tasmanian oyster supply chain but it could be easily modified for other states in Australia integrating their correspondent temperature-time data from the different oyster supply chain operations.

In conclusion, the *V. parahaemolyticus* models can help risk managers make objective decisions in exposure assessments. However, the models provide a “first estimate”. For this reason, persons using the models need to interpret correctly the output and understand models limitations. The tertiary model appeared to provide safe estimations and would be applicable as a public health tool. However, a high variability in the performance was observed and this should be considered. The models may be improved by adding other parameters apart from temperature. For example, the bacteria identified during the molecular studies may affect the growth of *V. parahaemolyticus* and could be included in

the tertiary model. A stochastic approach to modelling *V. parahaemolyticus* should be also considered as an alternative to reduce the present uncertainty and variability (e.g. differences among strains, microbial interactions). These predictive microbiology tools will be useful for oyster cold supply chain management, however further research can improve the accuracy of predictions.

## List of references

1. **Aaraas, R., I. J. Hernar, A. Vorre, H. Bergslien, B. T. Lunestad, S. Skeie, E. Slinde, and S. Mortensen.** 2004. Sensory, histological, and bacteriological changes in Flat oysters, *Ostrea edulis* L., during different storage conditions. *Journal of Food Science* **69**:205-210.
2. **Aarnisalo, K., S. Heiskanen, K. Jaakkola, E. Landor, and L. Raaska.** 2007. Traceability of foods and foodborne hazards. *VTT Research Notes* **2395**:52pp.
3. **Andrews, L. S., D. L. Park, and Y. P. Chen.** 2000. Low temperature pasteurization to reduce the risk of vibrio infections from raw shell-stock oysters. *Food additives and contaminants* **17**:787-791.
4. **Anonymous.** 2006. ASQAP Operations manual. Australian Shellfish Quality Assurance Advisory Committee.
5. **Anonymous.** 2009. Australian Fisheries Statistics 2008. ABARE and FRDC, Canberra, Australia.
6. **Anonymous.** 2005. Case study: Ring Ring, p. 30, Annual Report 2004/2005. NSW Food Authority
7. **Anonymous.** 2011. *Crassostrea gigas* (Thunberg, 1793) Fisheries and Aquaculture Department, FAO [http://www.fao.org/fishery/culturedspecies/Crassostrea\\_gigas/en](http://www.fao.org/fishery/culturedspecies/Crassostrea_gigas/en) [accessed 15/03/11].
8. **Anonymous.** 2009. Domestic fridge survey: summary. NSW Food Authority.
9. **Anonymous.** 1999. Food Safety Standards - Costs and Benefits. An analysis of the regulatory impact of the proposed national food safety reforms. FSANZ, Australia.
10. **Anonymous.** 2010. Guidelines on the application of general principles of food hygiene to the control of pathogenic *Vibrio* species in seafood CAC/GL 73-2010. Codex Alimentarius.
11. **Anonymous.** 2010. Joint FAO/WHO expert meeting on risk assessment tools for *Vibrio parahaemolyticus* and *Vibrio vulnificus* associated with seafoods. Call for data and experts. Deadline: 30 April 2010. FAO/WHO.
12. **Anonymous.** 1995. Multistate outbreak of viral gastroenteritis associated with consumption of oysters--Apalachicola Bay, Florida, December 1994-January 1995. *MMWR* **44**:37-39.

13. **Anonymous.** 2001. Opinion of the scientific committee on veterinary measures relating to public health on *Vibrio vulnificus* and *Vibrio parahaemolyticus*. European Commission, Health & consumer protection directorate-general
14. **Anonymous.** 2001. Outbreaks of Norwalk-like virus gastroenteritis linked to consumption of Pacific half shell oysters. New Zealand Public Health Report **8**:69.
15. **Anonymous.** 2011. PART 1.6 Microbiological and Processing Requirements, Standard 1.6.1 Microbiological limits for food. Australia New Zealand Food Standards Code
16. **Anonymous.** 2010. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food — 10 states, 2009. CDC. MMWR **59**:418-422.
17. **Anonymous.** 2005. Quantitative risk assessment on the public health impact of pathogenic *Vibrio parahaemolyticus* in raw oysters. USFDA, Washington, D.C.
18. **Anonymous.** 2011. *Saccostrea commercialis* (Iredale & Roughley, 1933) Fisheries and Aquaculture Department, FAO  
[http://www.fao.org/fishery/culturedspecies/Saccostrea\\_commercialis/es](http://www.fao.org/fishery/culturedspecies/Saccostrea_commercialis/es) [accessed 15/03/11].
19. **Anonymous.** 2006. Seafood supply chain innovations: Seafood traceability technologies. Seafood Services Australia.
20. **Anonymous.** 2007. Section II, Chapter VIII. Control of shellfish harvesting, National shellfish sanitation program guide for the control of molluscan shellfish. US-FDA and ISSC, Washington, D.C.
21. **Anonymous.** 2007. Section IV Chapter II. Bacteriological Examination of Shellfish Shipments Decision Tree, National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish. USFDA and ISSC, Washington, D.C.
22. **Anonymous.** 2007. Section IV, Chapter IV. Naturally occurring pathogens, National Shellfish Sanitation Program Guide for the Control of Molluscan Shellfish. US-FDA and ISSC, Washington, D.C.
23. **Anonymous.** 2006. *Vibrio parahaemolyticus* Infections Associated with Consumption of Raw Shellfish --- Three States, 2006. CDC. MMWR **55(Dispatch)**:1-2.
24. **Anonymous.** 2005. *Vibrio parahaemolyticus* Risk Assessment – Appendix 3: Risk Assessment Simulation Model. USFDA, Washington, D.C.
25. **Arrhenius, S.** 1915. Quantitative laws in biological chemistry. Bell G. and Sons Ltd, London.



26. **Ashie, I. N. A., J. P. Smith, and B. K. Simpson.** 1996. Spoilage and shelf-life extension of fresh fish and shellfish. *Critical Reviews in Food Science and Nutrition* **36**:87-121.
27. **Baert, K., K. Francois, B. De Meulenaer, and F. Devlieghere.** 2009. Risk assessment: a quantitative approach, p. 19-26. *In* R. Costa and K. Kristbergsson (ed.), *Predictive modeling and risk assessment*. Springer, USA.
28. **Baranyi, J., and T. A. Roberts.** 1994. A dynamic approach to predicting bacterial growth in food. *International Journal of Food Microbiology* **23**:277-294.
29. **Baranyi, J., and M. L. Tamplin.** 2004. ComBase: a common database on microbial responses to food environments. *Journal of Food Protection* **67**:1967-1971.
30. **Bej, A. K., D. P. Patterson, C. W. Brasher, M. C. L. Vickery, D. D. Jones, and C. A. Kaysner.** 1999. Detection of total and hemolysin-producing *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of *tl*, *tdh* and *trh*. *Journal of Microbiological Methods* **36**:215-225.
31. **Beleneva, I. A., N. V. Zhukova, H. Le-Lan, and D. H. Nguyen-Tran.** 2007. Taxonomic composition of bacteria associated with cultivated mollusks *Crassostrea lugubris* and *Perna viridis* and with the water of the Gulf of Nha Trang lagoon, Vietnam *Microbiology* **76**:220-228.
32. **Bentivoglio, M., and P. Pacini.** 1995. Filippo Pacini: A determined observer *Brain Research Bulletin* **38**:161-165.
33. **Bissett, A., J. P. Bowman, and C. Burke.** 2006. Bacterial diversity in organically-enriched fish farm sediments. *FEMS Microbiology Ecology* **55**:48-56.
34. **Blackstone, G. M., J. L. Nordstrom, M. C. L. Vickery, M. D. Bowen, R. F. Meyer, and A. DePaola.** 2003. Detection of pathogenic *Vibrio parahaemolyticus* in oyster enrichments by real time PCR. *Journal of Microbiological Methods* **53**:149-155.
35. **Blanco-Abad, V., J. Ansede-Bermejo, A. Rodriguez-Castro, and J. Martinez-Urtaza.** 2009. Evaluation of different procedures for the optimized detection of *Vibrio parahaemolyticus* in mussels and environmental samples. *International Journal of Food Microbiology* **129**:229-236.
36. **Bowman, J. P., S. A. McCammon, M. V. Brown, D. S. Nichols, and T. A. McMeekin.** 1997. Diversity and association of psychrophilic bacteria in Antarctic sea ice. *Applied and Environmental Microbiology* **63**:3068-3078.
37. **Broekaert, K., M. Heyndrickx, L. Herman, F. Devlieghere, and G. Vlaemynck.** 2011. Seafood quality analysis: Molecular identification of dominant microbiota

- after ice storage on several general growth media Food Microbiology **28**:1162-1169.
38. **Buchanan, R. L.** 1993. Developing and distributing user-friendly application software Journal of Industrial Microbiology & Biotechnology **12**:251-255.
39. **Buchanan, R. L.** 1993. Predictive food microbiology. Trends in Food Science & Technology **4**:6-11.
40. **Buchanan, R. L., and J. G. Phillips.** 2000. Updated models for the effects of temperature, initial pH, NaCl, and NaNO<sub>2</sub> on the aerobic and anaerobic growth of *Listeria monocytogenes* Quantitative microbiology **2**:103-128.
41. **Burnham, V. E., M. E. Janes, L. A. Jakus, J. Supan, A. DePaola, and J. Bell.** 2009. Growth and survival differences of *Vibrio vulnificus* and *Vibrio parahaemolyticus* strains during cold storage. Journal of Food Science **74**:314-318.
42. **Cao, R., C.-H. Xue, and Q. Liu.** 2009. Changes in microbial flora of Pacific oysters (*Crassostrea gigas*) during refrigerated storage and its shelf-life extension by chitosan. International Journal of Food Microbiology **131**:272-276.
43. **Cao, R., C.-H. Xue, Q. Liu, and Y. Xue.** 2009. Microbiological, chemical, and sensory assessment of Pacific oysters (*Crassostrea gigas*) stored at different temperatures. Czech Journal of Food Science **27**:102-108.
44. **Chae, M. J., D. Cheney, and Y.-C. Su.** 2009. Temperature effects on the depuration of *Vibrio parahaemolyticus* and *Vibrio vulnificus* from the American oyster (*Crassostrea virginica*). Journal of Food Science **74**:62-66.
45. **Colwell, R. R.** 1984. p. 48, Vibrios in the environment. John Wiley and Sons, New York.
46. **Colwell, R. R., and J. Liston.** 1960. Microbiology of Shellfish. Bacteriological study of the natural flora of Pacific oysters (*Crassostrea gigas*). Applied Microbiology **8**:104-109.
47. **Comiskey, S.** 2009. Australian oyster industry supply chain analysis. Project 2008/777. FRDC and CDI Pinnacle Management, Australia.
48. **Conaty, S., P. Bird, G. Bell, E. Kraa, G. Grohmann, and J. M. McAnulty.** 2000. Hepatitis A in New South Wales, Australia, from consumption of oysters: the first reported outbreak. Epidemiology and Infection **124**:121-130.
49. **Cook, D. W.** 1991. Microbiology of bivalve molluscan shellfish, p. 19-39. In D. R. Ward and C. R. Hackney (ed.), Microbiology of marine food products. Van Nostrand Reinhold, New York, London.

50. **Cook, D. W., P. O'Leary, J. C. Hunsucker, E. M. Sloan, J. C. Bowers, R. J. Blodgett, and A. Depaola.** 2002. *Vibrio vulnificus* and *Vibrio parahaemolyticus* in U.S. Retail Shell Oysters: A National Survey from June 1998 to July 1999 *Journal of Food Protection* **65**:79-87.
51. **Cook, D. W., and A. D. Ruple.** 1989. Indicator bacteria and *Vibrionaceae* multiplication in post-harvest shellstock oysters. *Journal of Food Protection* **52**:343-349.
52. **Croci, L., E. Suffredini, L. Cozzi, and L. Toti.** 2002. Effects of depuration of molluscs experimentally contaminated with *Escherichia coli*, *Vibrio cholerae* O1 and *Vibrio parahaemolyticus*. *Journal of Applied Microbiology* **92**:460-465.
53. **Dabanch, P. J., C. D. Herrero, A. C. Pavéz, P. N. Veas, J. S. Braun, and T. L. Porte.** 2009. *Vibrio parahaemolyticus* bacteremia: case report and literature review. *Revista chilena de infectologia* **26**:360-362.
54. **Dalgaard, P.** 2002. Modelling and predicting the shelf-life of seafood, p. 191-219. *In* H. A. Bremner (ed.), *Safety and quality issues in fish processing*. CRC Press LLC, USA.
55. **Dalgaard, P.** 1997. Predictive microbiological modelling and seafood quality, p. 431-443. *In* J. B. Luten, T. Borresen, and J. Oehlenschläger (ed.), *Seafood from producer to consumer, integrated approach to quality*. Elsevier, Amsterdam.
56. **Dalgaard, P., P. Buch, and S. Silberg.** 2002. Seafood Spoilage Predictor—development and distribution of a product specific application software *International Journal of Food Microbiology* **73**:343-349.
57. **Dalgaard, P., and L. V. Jorgensen.** 1998. Predicted and observed growth of *Listeria monocytogenes* in seafood challenge tests and in naturally contaminated cold-smoked salmon. *International Journal of Food Microbiology* **40**:105-115.
58. **Daniels, N. A., L. MacKinnon, R. Bishop, S. Altekruze, B. Ray, R. M. Hammond, S. Thompson, S. Wilson, N. H. Bean, P. M. Griffin, and L. Slutsker.** 2000. *Vibrio parahaemolyticus* infections in the United States, 1973-1998. *The journal of Infectious diseases* **18**:1661-1666.
59. **Davey, G. R.** 1985. Food poisoning in New South Wales: 1977-84. *Food Technology in Australia* **37**:453-457.
60. **Deepanjali, A., H. Sanath-Kumar, I. Karunasagar, and I. Karunasagar.** 2005. Seasonal variation in abundance of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters along the southwest coast of India. *Applied and Environmental Microbiology* **71**:3575-3580.

61. **DePaola, A., L. H. Hopkins, J. T. Peeler, B. Wentz, and R. M. McPhearson.** 1990. Incidence of *Vibrio parahaemolyticus* in U.S. coastal waters and oysters. *Applied and Environmental Microbiology* **56**:2299-2302.
62. **DePaola, A., J. L. Jones, J. Woods, W. Burkhardt III, K. R. Calci, J. A. Krantz, J. C. Bowers, K. Kasturi, R. H. Byars, E. Jacobs, D. Williams-Hill, and K. Nabe.** 2010. Bacterial and viral pathogens in live oysters: 2007 United States market survey. *Applied and Environmental Microbiology* **76**:2754-2768.
63. **DePaola, A., J. L. Nordstrom, J. C. Bowers, J. G. Wells, and D. W. Cook.** 2003. Seasonal abundance of total and pathogenic *Vibrio parahaemolyticus* in Alabama oysters. *Applied and Environmental Microbiology* **69**:1521-1526.
64. **Desmarchelier, P. M.** 2003. Pathogenic vibrios, p. 333-358. *In* A. D. Hocking (ed.), *Foodborne Microorganisms of Public Health Significance*, 6 ed. AIFST Inc., Waterloo.
65. **Desmarchelier, P. M.** 1978. *Vibrio parahaemolyticus* and other vibrios. *Food Technology in Australia* **30**:339-345.
66. **Devlieghere, F., K. Francois, B. De Meulenaer, and K. Baert.** 2006. Modelling food safety, p. 397-437. *In* P. A. Luning, F. Devlieghere, and R. Verhe (ed.), *Safety in the agri-food chain*. Wageningen Academic Publishers, Wageningen, The Netherlands.
67. **Devlieghere, F., K. Francois, A. Vermeulen, and J. Debevere.** 2009. Predictive microbiology, p. 29-53. *In* R. Costa and K. Kristbergsson (ed.), *Predictive modeling and risk assessment*. Springer, New York, USA.
68. **Devlieghere, F., A. H. Geeraerd, K. J. Versyck, B. Vandewaetere, J. Van Impeb, and J. Debeverea.** 2001. Growth of *Listeria monocytogenes* in modified atmosphere packed cooked meat products: a predictive model *Food Microbiology* **18**:53-66.
69. **Di Pinto, A., V. Terio, L. Novello, and G. Tantillo.** 2010. Comparison between thiosulfate-citrate-bile salt-sucrose (TCBS) agar and CHROMagar *Vibrio* for isolating *Vibrio parahaemolyticus*. *Food Control* **22**:124-127.
70. **Dileep, V., H. S. Kumar, Y. Kumar, M. Nishibuchi, I. Karunasagar, and I. Karunasagar.** 2003. Application of polymerase chain reaction for detection of *Vibrio parahaemolyticus* associated with tropical seafoods and coastal environment. *Letters in Applied Microbiology* **36**:423-427.
71. **Drake, S. L., A. DePaola, and L. Jaykus.** 2007. An overview of *Vibrio vulnificus* and *Vibrio parahaemolyticus*. *Comprehensive reviews in food science and food safety* **6**:120-144.

72. **Eyles, M. J., and G. R. Davey.** 1984. Microbiology of commercial depuration of the Sydney Rock oyster, *Crassostrea commercialis*. Journal of Food Protection **47**:703-706.
73. **Eyles, M. J., G. R. Davey, and G. Arnold.** 1985. Behavior and incidence of *Vibrio parahaemolyticus* in Sydney rock oysters (*Crassostrea commercialis*). International Journal of Food Microbiology **1**:327-334.
74. **FAO/WHO.** 2005. Risk assessment of choleraenic *Vibrio cholerae* 01 and 0139 in warm-water shrimp in international trade. Interpretative summary and technical report. Microbiological risk assessment series, vol. 9.
75. **FAO/WHO.** 2005. Risk assessment of *Vibrio vulnificus* in raw oysters. Interpretative summary and technical report. Microbiological risk assessment series, vol. 8.
76. **Farmer III, J. J.** 1979. *Vibrio* ("Benecke") *vulnificus*, the bacterium associated with sepsis, septicaemia, and the sea. Lancet **27**:903.
77. **Fleet, G. H., P. Heiskanen, I. Reid, and K. A. Buckle.** 2000. Foodborne viral illness - status in Australia. International Journal of Food Microbiology **59**:127-136.
78. **Frederiksen, M.** 2002. Quality chain management in fish processing, p. 289-307. In A. Bremner (ed.), Safety and quality issues in fish processing. Woodhead Publishing Limited and CRC Press LLC, USA.
79. **Frederiksen, M., C. Østerberg, S. Silberg, E. Larsen, and A. Bremner.** 2002. Info-Fisk. Development and validation of an internet based traceability system in a danish domestic fresh fish chain. Journal of Aquatic Food Product Technology **11**:13-34.
80. **Fujikawa, H., B. Kimura, and T. Fujii.** 2009. Development of a predictive program for *Vibrio parahaemolyticus* growth under various environmental conditions. Biocontrol Science **14**:127-131.
81. **Fujino, T., Y. Okuno, D. Nakada, A. Aoyama, K. Fukai, T. Mukai, and T. Ueho.** 1953. On the bacteriological examination of shirasu-food poisoning. Medical Journal of Osaka University **4**:299-304.
82. **García, K., R. Torres, P. Uribe, C. Hernández, M. L. Rioseco, J. Romero, and R. T. Espejo.** 2009. Dynamics of clinical and environmental *Vibrio parahaemolyticus* strains during seafood-related summer diarrhea outbreaks in southern Chile. Applied and Environmental Microbiology **75**:7482-7487.
83. **Garnier, M., Y. Labreuche, C. Garcia, M. Robert, and J. L. Nicolas.** 2007. Evidence for the involvement of pathogenic bacteria in summer mortalities of the Pacific oyster *Crassostrea gigas*. Microbial Ecology **53**:187-196.

84. **Gooch, J. A., A. DePaola, J. Bowers, and D. L. Marshall.** 2002. Growth and survival of *Vibrio parahaemolyticus* in postharvest American oysters. *Journal of Food Protection* **65**:970-974.
85. **Gooch, J. A., A. DePaola, C. A. Kaysner, and D. L. Marshall.** 2001. Evaluation of two direct plating methods using nonradioactive probes for enumeration of *Vibrio parahaemolyticus* in oysters. *Applied and Environmental Microbiology* **67**:721-724.
86. **Good, I. J.** 1953. The population frequencies of species and the estimation of population parameters. *Biometrika* **40**:237-264.
87. **Gosink, J. J., C. R. Woese, and J. T. Staley.** 1998. *Polaribacter* gen. nov., with three new species, *P. irgensii* sp. nov., *P. franzmannii* sp. nov. and *P. filamentus* sp. nov., gas vacuolate polar marine bacteria of the *Cytophaga-Flavobacterium-Bacteroides* group and reclassification of '*Flectobacillus glomeratus*' as *Polaribacter glomeratus* comb. nov. *International Journal of Systematic Bacteriology* **48**:223-235.
88. **Gosling, E.** 2003. An introduction to Bivalves, p. 1-6, *Bivalve Molluscs: Biology, Ecology and Culture*. Finishing New Books, Oxford, UK.
89. **Gram, L., and H. H. Huss.** 2000. Fresh and processed fish and shellfish, p. 472-506. In B. M. Lund, T. C. Baird-Parker, and G. W. Gould (ed.), *The microbiological safety and quality of food*. Aspen Publishers, Inc., Maryland, USA.
90. **Gram, L., and H. H. Huss.** 1996. Microbiological spoilage of fish and fish products. *International Journal of Food Microbiology* **33**:121-137.
91. **Gram, L., C. Wedell-Neergaard, and H. H. Huss.** 1990. The bacteriology of fresh and spoiling Lake Victorian Nile perch (*Lates niloticus*) *International Journal of Food Microbiology* **10**:303-316.
92. **Green, T. J., and A. C. Barnes.** 2010. Bacterial diversity of the digestive gland of Sydney rock oysters, *Saccostrea glomerata* infected with the paramyxean parasite, *Marteilia sydneyi*. *Journal of Applied Microbiology* **109**:613-622.
93. **Grohmann, G. S., H. B. Greenberg, B. M. Welch, and A. M. Murphy.** 1980. Oyster-Associated gastroenteritis in australia: the detection of norwalk virus and its antibody by immune electron microscopy and radioimmunoassay. *Journal of Medical Virology* **6**:11-19.
94. **Hahn, M. W., E. Lang, U. Brandt, and C. Spröer.** 2011. *Polynucleobacter acidiphobus* sp. nov., a representative of an abundant group of planktonic freshwater bacteria. *International Journal of Systematic and Evolutionary Microbiology* **61**:788-794.

95. **Hall, T. A.** 1999. Bio Edit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series **41**:95-98.
96. **Hamady, M., C. Lozupone, and R. Knight.** 2010. Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. The ISME Journal **4**:17-27.
97. **Hara-Kudo, Y., T. Nishina, H. Nakagawa, H. Konuma, J. Hasegawa, and S. Kumagai.** 2001. Improved Method for Detection of *Vibrio parahaemolyticus* in Seafood Applied and Environmental Microbiology **67**:5819-5823.
98. **Harth, E., L. Matsuda, C. Hernández, M. L. Rioseco, J. Romero, N. González-Escalona, J. Martínez-Urtaza, and R. T. Espejo.** 2009. Epidemiology of *Vibrio parahaemolyticus* outbreaks, Southern Chile. Emerging Infectious Diseases **15**:163-168.
99. **Hernández-Zárate, G., and J. Olmos-Soto.** 2006. Identification of bacterial diversity in the oyster *Crassostrea gigas* by fluorescent in situ hybridization and polymerase chain reaction. Journal of Applied Microbiology **100**:664-672.
100. **Honda, S., I. Goto, I. Minematsu, N. Ikeda, N. Asano, M. Ishibashi, Y. Kinoshita, M. Nishibuchi, T. Honda, and T. Miwatani.** 1987. Gastroenteritis Due to Kanagawa Negative *Vibrio parahaemolyticus*. Lancet **1**:331-332.
101. **Honda, T., K. Goshima, Y. Takeda, Y. Sugino, and T. Miwatani.** 1976. Demonstration of the cardiotoxicity of the thermostable direct hemolysin (lethal toxin) produced by *Vibrio parahaemolyticus*. . Infection and Immunity **13**:163-171.
102. **Honda, T., T. Iida, Y. Akeda, and T. Kodama.** 2008. Sixty years of *Vibrio parahaemolyticus* research. Microbe **3**:462-466.
103. **Honda, T., Y. X. Ni, and T. Miwatani.** 1988. Purification and characterization of a hemolysin produced by a clinical isolate of Kanagawa phenomenon-negative *Vibrio parahaemolyticus* and related to the thermostable direct hemolysin. Infection and Immunity **56**:961-965.
104. **Hood, M. A., G. E. Ness, G. E. Rodrick, and N. J. Blake.** 1983. Effects of storage on microbial loads of two commercially important shellfish species, *Crassostrea virginica* and *Mercenaria campechiensis*. Applied and Environmental Microbiology **45**:1221-1228.
105. **Hunt, D. A., J. Miescier, J. Redman, A. Salinger, and J. P. Lucas.** 1984. Molluscan shellfish, fresh or fresh frozen oysters, mussels, or clams. In M. L. Speck (ed.), Compendium of methods for the microbiological examination of foods, 2nd ed. American Public Health Association, Washington D.C.

106. **Hunter, A. C., and B. A. Linden.** 1923. An investigation of oyster spoilage. The American Food Journal **18**:538-540.
107. **Huss, H. H.** 1994. Assurance of seafood quality. FAO fisheries technical paper 334 Rome, Italy.
108. **Jagoe, C.** 2011. Survey of bacterial communities found in the Eastern oyster (*Crassostrea virginica*) microbiome, The American Society for Microbiology 2011, 21-24 May, New Orleans, USA [conference abstract].
109. **Jarvis, B. D. W., P. Van Berkum, W. X. Chen, S. M. Nour, M. P. Fernandez, J. C. Cleyet-Marel, and M. Gillis.** 1997. Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. International Journal of Systematic and Evolutionary Microbiology **47**:895-898.
110. **Jay, J. M.** 2000. Chapter 20. Indicators of food microbial quality and safety, p. 387-405. In D. R. Heldman (ed.), Modern food microbiology 6th Edition. Aspen Publishers, Inc., Maryland, USA.
111. **Johnson, C. N., A. R. Flowers, N. F. Noriega III, A. M. Zimmerman, J. C. Bowers, A. DePaola, and D. J. Grimes.** 2010. Relationships between environmental factors and pathogenic *Vibrios* in the Northern Gulf of Mexico. Applied and Environmental Microbiology **76**:7076-7084.
112. **Johnson, W. G., A. C. Salinger, and W. C. King.** 1973. Survival of *Vibrio parahaemolyticus* in oyster shellstock at two different storage temperatures. Applied Microbiology **26**:122-123.
113. **Kaufman, G. E., A. K. Bej, J. Bowers, and A. DePaola.** 2003. Oyster-to-oyster variability in levels of *Vibrio parahaemolyticus*. Journal of Food Protection **66**:125-129.
114. **Kaysner, C. A.** 2000. *Vibrio* species, p. 1337-1362. In B. M. Lund, T. C. Baird-Parker, and G. W. Gould (ed.), The microbiological safety and quality of food, vol. 2. Aspen Publishers, Inc., Gaithersburg, USA.
115. **Kaysner, C. A., and A. DePaola.** 2004. *Vibrio*, Bacteriological Analytical Manual. US-FDA, Washington DC.
116. **Kaysner, C. A., M. L. Tamplin, M. M. Wekell, R. F. Stott, and K. G. Colburn.** 1989. Survival of *Vibrio vulnificus* in shellstock and shucked oysters (*Crassostrea gigas* and *Crassostrea virginica*) and effects of isolation medium on recovery. Applied and Environmental Microbiology **55**:3072-3079.
117. **Kennedy, J., V. Jackson, S. Blair, D. A. McDowell, C. Cowan, and D. J. Bolton.** 2005. Food safety knowledge of consumers and the microbiological and temperature status of their refrigerators. Journal of Food Protection **68**:1421-1430.



118. **Kim, Y.-M., H.-D. Payk, and D.-S. Lee.** 2002. Shelf-life characteristics of fresh oysters and ground beef as affected by bacteriocin-coated plastic packaging film. *Journal of Science of Food of Agriculture* **82**:998-1002.
119. **Kraa, E.** 1995. Surveillance and epidemiology of foodborne illness in NSW, Australia. *Food Australia* **47**:418-423.
120. **Kueh, C. S., and K. Y. Chan.** 1985. Bacteria in bivalve shellfish with special reference to the oyster. *Journal of Applied Bacteriology* **59**:41-47.
121. **Kuznesof, S., and M. Brennan.** 2007. Perceived risk and product safety in the food supply chain, p. 32-47. *In* M. A. Bourlakis and P. W. H. Weightman (ed.), *Food supply chain management*. Blackwell Publishing Ltd., Oxford.
122. **Kwon, K. K., and S.-J. Kim.** 2010. Marine, hydrocarbon-degrading *Alphaproteobacteria*, p. 1707-1714. *In* K. N. Timmis (ed.), *Handbook of hydrocarbon and lipid microbiology*. Springer, Berlin, Heidelberg.
123. **Lee, C. Y., M. F. Cheng, M. S. Yu, and M. J. Pan.** 2002. Purification and characterization of a putative virulence factor, serine protease, from *Vibrio parahaemolyticus*. *FEMS Microbiology Letters* **209**:31-37.
124. **Lees, D.** 2000. Viruses and bivalve shellfish. *International Journal of Food Microbiology* **59**:81-116.
125. **Lewis, T., M. Brown, G. Abell, T. A. McMeekin, and J. Summner.** 2003. Pathogenic *Vibrio parahaemolyticus* in Australian oysters. Project 2002/409. FRDC and University of Tasmania, Hobart, Australia.
126. **Liu, C., J. Lu, and Y. Su.** 2009. Effects of flash freezing, followed by frozen storage, on reducing *Vibrio parahaemolyticus* in pacific raw oysters (*Crassostrea gigas*). *Journal of Food Protection* **72**:174-177.
127. **Liu, W. T., T. L. Marsh, H. Cheng, and L. J. Forney.** 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Applied and Environmental Microbiology* **63**:4516-4522.
128. **Lopez-Lopez, A., M. J. Pujalte, S. Benlloch, M. Mata-Roig, R. Rossello-Mora, E. Garay, and F. Rodriguez-Valera.** 2002. *Thalassospira lucentensis* gen. nov., sp. nov., a new marine member of the *alpha-Proteobacteria*. *International Journal of Systematic and Evolutionary Microbiology* **52**:1277-1283.
129. **Lorca, T. A., M. D. Pierson, G. J. Flick, and C. R. Hackney.** 2001. Levels of *Vibrio vulnificus* and organoleptic quality of raw shellstock oysters (*Crassostrea virginica*) maintained at different storage temperatures. *Journal of Food Protection* **64**:1716-1721.

130. **Lozano-León, A., J. Torres, C. R. Osorio, and J. Martínez-Urtaza.** 2003. Identification of tdh-positive *Vibrio parahaemolyticus* from an outbreak associated with raw oyster consumption in Spain FEMS Microbiology Letters **226** 281-284
131. **Ma, L., and Y. C. Su.** 2011. Validation of high pressure processing for inactivating *Vibrio parahaemolyticus* in Pacific oysters (*Crassostrea gigas*). International Journal of Food Microbiology **144**:469-474.
132. **Madigan, T. L.** 2008. A critical evaluation of supply-chain temperature profiles to optimise food safety and quality of Australian oysters. Seafood CRC and SARDI.
133. **Madigan, T. L., K. J. Lee, A. M. Pointon, and C. J. Thomas.** 2007. A supply-chain assessment of marine *Vibrios* in Pacific oysters in South Australia: prevalence, quantification and public health risk. Project 2005/401. FRDC and Univeristy of Adelaide, Adelaide, Australia.
134. **Maguire, G. B., and J. A. Nell.** 2007. History, Status and Future of Oyster Culture in Australia. The 1st International Oyster Symposium Proceedings, Tokyo, Japan, p. 3-12, Oyster Research Institute News, vol. 19.
135. **Mahmoud, B. S. M., and D. D. Burrage.** 2009. Inactivation of *Vibrio parahaemolyticus* in pure culture, whole live and half shell oysters (*Crassostrea virginica*) by X-ray. Letters in Applied Microbiology **48**:572-578.
136. **Margulis, L., L. Nault, and J. M. Sieburth.** 1991. *Cristispira* from oyster styles: complex morphology of large symbiotic *spirochetes*. Symbiosis **11**:1-17.
137. **Marklinder, I. M., M. Lindblad, L. M. Eriksson, A. M. Finnson, and R. Lindqvist.** 2004. Home storage temperatures and consumer handling of refrigerated foods in Sweden. Journal of Food Protection **67**:2570-2577.
138. **Marques, A., M. L. Nunes, S. K. Moore, and M. S. Strom.** 2010. Climate change and seafood safety: Human health implications Food Research International **43**:1766-1779.
139. **Marquez, L., A. Higgins, and S. Estrada-Flores.** 2010. Understanding Victoria's Fruit and Vegetable Freight Movements. Victorian Eco-innovation lab, University of Melbourne:1-170.
140. **Martinez-Urtaza, J., J. C. Bowers, J. Trinanes, and A. DePaola.** 2010. Climate anomalies and the increasing risk of *Vibrio parahaemolyticus* and *Vibrio vulnificus* illnesses Food Research International **43**:1780-1790.
141. **Martinez-Urtaza, J., B. Huapaya, R. G. Gavilan, V. Blanco-Abad, J. Ansedo-Bermejo, C. Cadarso-Suarez, A. Figueiras, and J. Trinanes.** 2008. Emergence of Asiatic *Vibrio* diseases in South America in phase with El Niño. Epidemiology **19**:829-837.

142. **Martinez-Urtaza, J., L. Simental, D. Velasco, A. DePaola, M. Ishibashi, Y. Nakaguchi, M. Nishibuchi, D. Carrera-Flores, C. Rey-Alvarez, and A. Pousa.** 2005. Pandemic *Vibrio parahaemolyticus* O3:K6, Europe. *Emerging Infectious Diseases* **11**:1319-1320.
143. **McDonald, K., and D. Sun.** 1999. Predictive food microbiology for the meat industry: a review. *International Journal of Food Microbiology* **52**:1-27.
144. **Mckellar, R. C., and X. Lu.** 2000. Primary models, p. 21-62. *In* R. C. Mckellar and X. Lu (ed.), *Modeling microbial responses in food*. CRC Press, Boca Raton, USA.
145. **McMeekin, T.** 2000. An essay on the unrealized potential of predictive microbiology, p. 321-335. *In* R. C. Mckellar and X. Lu (ed.), *Modeling microbial responses in food*. CRC Press LLC, Boca Raton, USA.
146. **McMeekin, T.** 2007. Predictive microbiology: past, present and future, p. 7-19. *In* S. Brul, S. van Gerwen, and M. H. Zwietering (ed.), *Modelling microorganisms in food*. Woodhead Publishing Limited and CRC Press LLC, USA.
147. **McMeekin, T., J. Bowman, O. McQuestin, L. Mellefont, T. Ross, and M. L. Tamplin.** 2008. The future of predictive microbiology: Strategic research, innovative applications and great expectations. *International Journal of Food Microbiology* **128**:2-9.
148. **McMeekin, T., J. Olley, D. A. Ratkowsky, and T. Ross.** 2002. Predictive microbiology: towards the interface and beyond. *International Journal of Food Microbiology* **73**:395-407.
149. **McMeekin, T., J. Olley, T. Ross, and D. A. Ratkowsky.** 1993. *Predictive microbiology: theory and application*. Research studies press Ltd., Taunton.
150. **McMeekin, T., and T. Ross.** 2002. Predictive microbiology: providing a knowledge-based framework for change management *International Journal of Food Microbiology* **78**:133-153.
151. **McMeekin, T., N. Smale, I. Jenson, T. Ross, and D. Tanner (ed.).** 2006. Combining microbial growth models with near real-time temperature monitoring technologies to estimate the shelf life and safety of foods during processing and distribution. *Proceedings of the 2nd international Workshop Cold Chain Management* 8-9 May. Kreyenschmidt, J. & B. Petersen Bonn.
152. **McMeekin, T. A., J. Baranyi, J. Bowman, P. Dalgaard, M. Kirk, T. Ross, S. Schimide, and M. H. Zwietering.** 2006. Information systems in food safety management *International Journal of Food Microbiology* **112**:181-194.

153. **Mejlholm, O., and P. Dalgaard.** 2007. Modeling and predicting the growth of lactic acid bacteria in lightly preserved seafood and their inhibiting effect on *Listeria monocytogenes*. *Journal of Food Protection* **70**:2485-2497.
154. **Michael, K., and L. McCathie.** 2005. The pros and cons of RFID in supply chain management. University of Wollongong. <http://ro.uow.edu.au/infopapers/105/> [accessed 24/02/11].
155. **Miles, D. W., T. Ross, J. Olley, and T. A. McMeekin.** 1997. Development and evaluation of a predictive model for the effect of temperature and water activity on the growth rate of *Vibrio parahaemolyticus*. *International Journal of Food Microbiology* **38**:133-142.
156. **Miyamoto, Y., T. Kato, Y. Obara, S. Akiyama, K. Takizawa, and S. Yamai.** 1969. In vitro hemolytic characteristic of *Vibrio parahaemolyticus* - its close correlation with human pathogenicity. *Journal of Bacteriology* **100**:1147-&.
157. **Najiah, M., M. Nadirah, K. L. Lee, S. W. Lee, W. Wendy, H. H. Ruhil, and F. A. Nurul.** 2008. Bacteria flora and heavy metals in cultivated oysters *Crassostrea iredalei* of Setiu Wetland, East Coast Peninsular Malaysia *Veterinary research communications* **32**:377-381.
158. **Nauta, M. J.** 2002. Modelling bacterial growth in quantitative microbiological risk assessment: is it possible? *International Journal of Food Microbiology* **73**:297-304.
159. **Nauta, M. J.** 2008. The modular process risk model (MPRM): a structured approach to food chain exposure assessment, p. 99-136. *In* D. W. Schaffner (ed.), *Microbial risk analysis of foods*. ASM Press, Washington D.C.
160. **Nauta, M. J.** 2000. Separation of uncertainty and variability in quantitative microbial risk assessment models. *International Journal of Food Microbiology* **57**:9-18.
161. **Nauta, M. J.** 2007. Uncertainty and variability in predictive models of microorganisms in foods, p. 44-66. *In* S. Brul, S. Van Gerwen, and M. H. Zwietering (ed.), *Modelling microorganisms in food*. Woodhead Publishing Limited and CRC Press LLC, USA.
162. **Nauta, M. J., S. Litman, G. C. Barker, and F. Carlin.** 2003. A retail and consumer phase model for exposure assessment of *Bacillus cereus* *International Journal of Food Microbiology* **83**:205-218.
163. **Navarrete, P., R. T. Espejo, and J. Romero.** 2009. Molecular analysis of microbiota along the digestive tract of juvenile Atlantic salmon (*Salmo salar* L.) *Microbial Ecology* **57**:550-561.
164. **Nell, J. A.** 1993. Farming the Sydney rock oyster (*Saccostrea commercialis*) in Australia. *Reviews in Fisheries Science* **1**:97-120.

165. **Nell, J. A., and P. J. Gibbs.** 1986. Salinity tolerance and absorption of L-Methionine by some Australian bivalve molluscs. *Australian Journal of Marine and Freshwater Research* **37**:721-727.
166. **Nilsson, L., and L. Gram.** 2002. Improving the control of pathogens in fish products, p. 54-84. *In* A. Bremner (ed.), *Safety and quality issues in fish processing*. Woodhead publishing limited and CRC Press LLC, Boca Raton, USA.
167. **Nishibuchi, M., and J. B. Kaper.** 1985. Nucleotide sequence of the thermostable direct hemolysin gene of *Vibrio parahaemolyticus*. *Journal of Bacteriology* **162**:558-564.
168. **Nishibuchi, M., T. Taniguchi, T. Misawa, V. Khaeomanee-iam, T. Honda, and T. Miwatani.** 1989. Cloning and nucleotide-sequence of the gene (trh) encoding the hemolysin related to the thermostable direct hemolysin of *Vibrio parahaemolyticus*. *Infection and Immunity* **57**:2691-2697.
169. **Nordstrom, J. L., M. C. L. Vickery, G. M. Blackstone, S. L. Murray, and A. DePaola.** 2007. Development of a multiplex real-time PCR assay with an internal amplification control for the detection of total and pathogenic *Vibrio parahaemolyticus* bacteria in oysters. *Applied and Environmental Microbiology* **73**:5840-5847.
170. **Nuin, M., B. Alfaro, Z. Cruz, N. Argarate, S. George, Y. Le March, J. Olley, and C. Pin.** 2008. Modelling spoilage of fresh turbot and evaluation of a time-temperature integrator (TTI) label under fluctuating temperature *International Journal of Food Microbiology* **127**:193-199
171. **Oertli, G. E., C. Jenkins, N. Ward, F. Rainey, E. Stackebrandt, and J. T. Staley.** 2006. The genera *Prosthecomicrobium* and *Ancalomicrobium*, p. 65-71. *In* M. Dworkin, S. Falkow, E. Rosenberg, K.-H. Schleifer, and E. Stackebrandt (ed.), *The Prokaryotes. Vol. 5: Proteobacteria: Alpha and Beta Subclass*. Springer, New York, USA.
172. **Oliveira, J., A. Cunha, F. Castilho, J. L. Romalde, and M. J. Pereira.** 2011. Microbial contamination and purification of bivalve shellfish: Crucial aspects in monitoring and future perspectives – A mini-review *Food Control* **22**:805-816.
173. **Oliver, J. D., and J. B. Kaper.** 2007. Part III. Foodborne Pathogenic Bacteria. 16. *Vibrio* species p. 343-379 *In* M. P. Doyle and L. R. Beuchat (ed.), *Food Microbiology. Fundamentals and Frontiers*, 3 ed. ASM Press, Washington, D.C.
174. **Orcutt, B., B. Bailey, H. Staudigel, B. M. Tebo, and K. J. Edwards.** 2009. An interlaboratory comparison of 16S rRNA gene-based terminal restriction fragment length polymorphism and sequencing methods for assessing microbial diversity of seafloor basalts. *Environmental Microbiology* **11**:1728-1735.

175. **Otsuka, T., T. Noda, A. Noguchi, H. Nakamura, K. Ibaraki, and K. Yamaoka.** 2007. *Shewanella* infection in decompensated liver disease: a septic case. *Journal of Gastroenterology* **42**:87-90.
176. **Parveen, S., K. A. Hettiarachchi, J. C. Bowers, J. L. Jones, M. L. Tamplin, R. McKay, W. Beatty, K. Brohawn, L. V. DaSilva, and A. DePaola.** 2008. Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. *International Journal of Food Microbiology* **128**:354-361.
177. **Payinda, G.** 2008. Necrotizing fasciitis due to *Vibrio parahaemolyticus*. *New Zealand medical journal* **121**:99-101.
178. **Phillips, A. M. B., A. DePaola, J. Bowers, S. Ladner, and D. J. Grimes.** 2007. An evaluation of the use of remotely sensed parameters for prediction of incidence and risk associated with *Vibrio parahaemolyticus* in Gulf Coast Oysters (*Crassostrea virginica*). *Journal of Food Protection* **70**:879-884.
179. **Poiulot, R., M. B. Lubran, S. C. Cates, and S. Dennis.** 2010. Estimating parametric distributions of storage time and temperature of ready-to-eat foods for U.S. households. *Journal of Food Protection* **73**:312-321.
180. **Potasman, I., A. Paz, and M. Odeh.** 2002. Infectious outbreaks associated with bivalve shellfish consumption: A worldwide perspective. *Clinical Infectious Diseases* **35**:921-928.
181. **Powell, S. M., J. S. Stark, I. Snape, E. N. M. Woolfenden, J. P. Bowman, and M. J. Riddle.** 2010. Effects of diesel and lubricant oils on Antarctic benthic microbial communities over five years. *Aquatic Microbial Ecology* **61**:119-127.
182. **Pujalte, M. J., M. Ortigosa, M. C. Macian, and E. Garay.** 1999. Aerobic and facultative anaerobic heterotrophic bacteria associated to Mediterranean oysters and seawater. *International Microbiology* **2**:259-266.
183. **Raimondi, F., J. P. Y. Kao, C. Fiorentini, A. Fabbri, G. Donelli, N. Gasparini, A. Rubino, and A. Fasano.** 2000. Enterotoxicity and cytotoxicity of *Vibrio parahaemolyticus* thermostable direct hemolysin in in vitro systems *Infection and Immunity* **68**:3180-3185.
184. **Rasch, M.** 2004. Experimental design and data collection, p. 1-20. *In* R. C. Mckellar and X. Lu (ed.), *Modeling microbial responses in food*. CRC Press, Boca Raton, USA.
185. **Ratkowsky, D. A.** 2004. Model fitting and uncertainty, p. 151-196. *In* R. C. Mckellar and X. Lu (ed.), *Modeling microbial responses in food*. CRC Press, Boca Raton, USA.

186. **Ratkowsky, D. A., J. Olley, T. A. McMeekin, and A. Ball.** 1982. Relationship between temperature and growth rate of bacterial cultures. *Journal of Bacteriology* **149**:1-5.
187. **Rees, G. N., D. S. Baldwin, G. O. Watson, S. Perryman, and D. L. Nielsen.** 2004. Ordination and significance testing of microbial community composition derived from terminal restriction fragment length polymorphisms: application of multivariate statistics *Antonie van Leeuwenhoek* **86**:339-347.
188. **Richards, G. P.** 1991. Shellfish depuration, p. 395-428. *In* D. A. Ward and C. Hackney (ed.), *Microbiology of marine food products*. Van Nostrand Reinhold, New York.
189. **Richards, G. P., M. A. Watson, E. J. Crane III, I. G. Burt, and D. Bushek.** 2008. *Shewanella* and *Photobacterium* spp. in oysters and seawater from the Delaware Bay. *Applied and Environmental Microbiology* **74**:3323-3327.
190. **Robertson, L. J.** 2007. Review. The potential for marine bivalve shellfish to act as transmission vehicles for outbreaks of protozoan infections in humans: A review. *International Journal of Food Microbiology* **120**:201-216.
191. **Romero, J., and R. T. Espejo.** 2001. The prevalence of non-cultivable bacteria in oysters (*Tiostrea chilensis*, Philippi, 1845). *Journal of Shellfish Research* **20**:1235-1240.
192. **Romero, J., M. Garcia-Varela, J. P. Laclette, and R. T. Espejo.** 2002. Bacterial 16SrRNA gene analysis revealed that bacteria related to *Arcobacter* spp. constitute an abundant and common component of the oyster microbiota (*Tiostrea chilensis*). *Microbial Ecology* **44**:365-371.
193. **Romero, J., N. González, and R. T. Espejo.** 2002. Marine *Pseudoalteromonas* sp. composes most of the bacterial population developed in oysters (*Tiostrea chilensis*) spoiled during storage. *Journal of Food Science* **67**:2300-2303.
194. **Ross, T.** 1996. Indices for performance evaluation of predictive models in food microbiology. *Journal of Applied Microbiology* **81**:501-508.
195. **Ross, T.** 2008. Microbial ecology in food safety risk assessment, p. 51-97. *In* D. W. Schaffner (ed.), *Microbial risk analysis of foods*. American society for microbiology ASM Press, Washington D.C.
196. **Ross, T., and P. Dalgaard.** 2000. Secondary models, p. 63-149. *In* R. C. Mckellar and X. Lu (ed.), *Modeling microbial responses in food*. CRC Press, Boca Raton, USA.
197. **Ross, T., P. Dalgaard, and S. Tienungoon.** 2000. Predictive modelling of the growth and survival of *Listeria* in fishery products *International Journal of Food Microbiology* **62**:231-245.

198. **Rossi, P., F. Gillet, E. Rohbach, N. Diaby, and C. Holliger.** 2009. Statistical assessment of variability of terminal restriction fragment length polymorphism analysis applied to complex microbial communities. *Applied and Environmental Microbiology* **75**:7268-7270.
199. **Sakurai, J., A. Matsuzaki, and T. Miwatani.** 1973. Purification and characterization of thermostable direct hemolysin of *Vibrio parahaemolyticus*. *Infection and Immunity* **8**:775-780.
200. **Satomi, M., B. Kimura, T. Hamada, S. Harayama, and T. Fujii.** 2002. Phylogenetic study of the genus *Oceanospirillum* based on 16S rRNA and gyrB genes: emended description of the genus *Oceanospirillum*, description of *Pseudospirillum* gen. nov., *Oceanobacter* gen. nov. and *Terasakiella* gen. nov. and transfer of *Oceanospirillum jannaschii* and *Pseudomonas stanieri* to *Marinobacterium* as *Marinobacterium jannaschii* comb. nov. and *Marinobacterium stanieri* comb. no. *International Journal of Systematic and Evolutionary Microbiology* **52**:739-747.
201. **Schothorst, M., M. H. Zwietering, T. Ross, R. L. Buchanan, and M. B. Cole.** 2009. Relating microbiological criteria to food safety objectives and performance objectives. *Food Control* **20**:967-979.
202. **Schuett, C., and H. Doepke.** 2010. Endobiotic bacteria and their pathogenic potential in cnidarian tentacles. *Helgoland marine research* **64**:205-212.
203. **Seaman, M. N. L.** 1991. Survival and aspects of metabolism in oysters, *Crassostrea gigas*, during and after prolonged air storage. *Aquaculture* **93**:389-395.
204. **Sekiguchi, H., M. Watanabe, T. Nakahara, B. Xu, and H. Uchiyama.** 2002. Succession of bacterial community structure along the Changjiang river determined by denaturing gradient gel electrophoresis and clone library analysis *Applied and Environmental Microbiology* **68**:5142-5150.
205. **Shen, X., Y. Cai, C. Liu, W. Liu, Y. Hui, and Y. C. Su.** 2009. Effect of temperature on uptake and survival of *Vibrio parahaemolyticus* in oysters (*Crassostrea plicatula*). *International Journal of Food Microbiology* **136**:129-132.
206. **Shiflett, M. A., J. S. Lee, and R. O. Sinnhuber.** 1966. Microbial flora of irradiated Dungeness crabmeat and Pacific oysters. *Applied Microbiology* **14**:411-415.
207. **Smith, D., and L. Sparks.** 2007. Temperature controlled supply chains, p. 179-198. *In* M. A. Bourlakis and P. W. H. Weightman (ed.), *Food supply chain management*. Blackwell Publishing Ltd., Oxford.
208. **Su, H. P., S. I. Chiu, J. L. Tsai, C. L. Lee, and T. M. Pan.** 2005. Bacterial food-borne illness outbreaks in northern Taiwan, 1995-2001. *Journal of Infection and Chemotherapy* **11**:146-151.



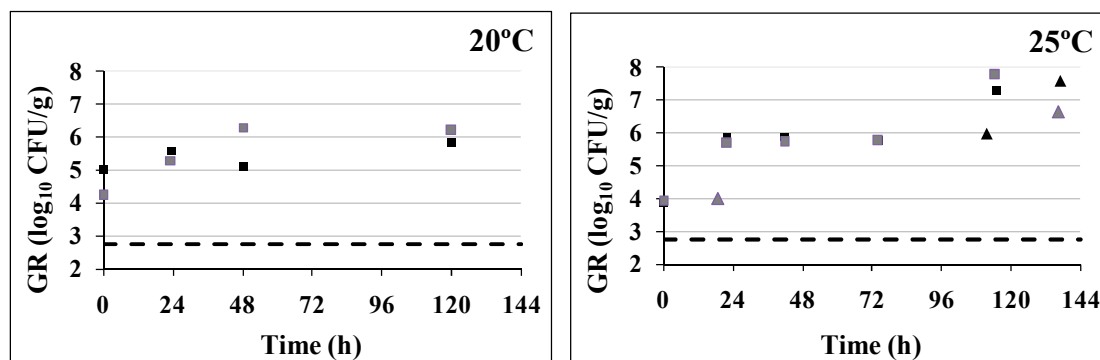
209. **Su, Y.-C., and C. Liu.** 2007. *Vibrio parahaemolyticus*: A concern of seafood safety. Food Microbiology **24**:549-558.
210. **Tamplin, M. L., J. Baranyi, and G. Paoli.** 2000. Software programs to increase the utility of predictive microbiology information, p. 233-242. In R. C. Mckellar and X. Lu (ed.), Modelling microbial responses in food. CRC Press, Boca Raton, USA.
211. **Tamplin, M. L., and G. M. Capers.** 1992. Persistence of *Vibrio vulnificus* in tissues of gulf coast oysters, *Crassostrea virginica*, exposed to seawater disinfected with UV light. Applied and Environmental Microbiology **58**:1506-1510.
212. **Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar.** 4 May 2011, published online. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. doi: 10.1093/molbev/msr121
213. **Taniguchi, H., H. Ohta, M. Ogawa, and Y. Mizuguchi.** 1985. Cloning and expression in *Escherichia coli* of *Vibrio parahaemolyticus* thermostable direct hemolysin and thermolabile hemolysin genes. Journal of Bacteriology **162**:510-515.
214. **Taoukis, P. S., K. Koutsoumanis, and G. J. E. Nychas.** 1999. Use of time-temperature integrators and predictive modelling for shelf life control of chilled fish under dynamic storage conditions. International Journal of Food Microbiology **53**:21-31.
215. **Taoukis, P. S., and T. P. Labuza.** 2003. Time-temperature indicators (TTI), p. 103-126. In R. Ahvenainen (ed.), Novel food packaging techniques. Woodhead Publishing Limited, UK.
216. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acid Research **11**:4673-4680.
217. **Trienekens, J., and J. van der Vorst.** 2006. Traceability in food supply chains, p. 439-470. In P. A. Luning, F. Devlieghere, and R. Verhe (ed.), Safety in the agri-food chain. Wageningen Academic Publishers, Wageningen.
218. **Tsironi, T., A. Stamatiou, M. Giannoglou, E. Velliou, and P. S. Taoukis.** 2011. Predictive modelling and selection of Time Temperature Integrators for monitoring the shelf life of modified atmosphere packed gilthead seabream fillets LWT-Food Science and Technology **44**:1156-1163.
219. **Tunung, R., F. M. Ghazali, M. A. Noranizan, K. K. Haresh, M. B. Lesley, Y. Nakaguchi, M. Nishibuchi, and R. Son.** 2011. Rapid detection and enumeration

- of pathogenic *Vibrio parahaemolyticus* in raw vegetables from retail outlets. International Food Research Journal **18**:67-78.
220. **Vannini, C., G. Petroni, F. Verni, and G. Rosati.** 2005. *Polynucleobacter* bacteria in the brackish-water species *Euplotes harpa* (Ciliata Hypotrichia). Journal of Eukaryotic Microbiology **52**:116-122.
  221. **Vasconcelos, G. J., and J. S. Lee.** 1972. Microbial flora of Pacific oysters (*Crassostrea gigas*) subjected to ultraviolet-irradiated seawater. Applied Microbiology **23**:11-16.
  222. **Vongxay, K., S. Wang, X. Zhang, B. Wu, H. Hu, Z. Pan, S. Chen, and W. Fang.** 2008. Pathogenetic characterization of *Vibrio parahaemolyticus* isolates from clinical and seafood sources. International Journal of Food Microbiology **126**:71-75.
  223. **Vose, D.** 1996. Quantitative risk analysis: a guide to Monte Carlo simulation modelling. John Willey & sons Ltd, England.
  224. **Vose, D.** 2008. Risk Analysis: A Quantitative Guide 3rd edition. John Wiley & sons Ltd., England.
  225. **Wang, D., S. Yu, W. Chen, D. Zhang, and X. Shi.** 2010. Enumeration of *Vibrio parahaemolyticus* in oyster tissues following artificial contamination and depuration. Letters in Applied Microbiology **51**:104-108.
  226. **Webby, R. J., K. S. Carville, M. D. Kirk, G. Greening, R. M. Ratcliff, S. K. Crerar, K. Dempsey, M. Sarna, R. Stafford, M. Patel, and G. Hall.** 2007. Internationally distributed frozen oyster meat causing multiple outbreaks of norovirus infection in Australia. Clinical Infectious Diseases **44**:1026-1031.
  227. **Weis, K. E., R. M. Hammond, R. Hutchinson, and G. M. Blackmore.** 2011. *Vibrio* illness in Florida, 1998–2007. Epidemiology and Infection **139**:591-598.
  228. **Westrell, T., V. Dusch, S. Ethelberg, J. Harris, M. Hjertqvist, N. J. da Silva, A. Koller, A. Lenglet, M. Lisby, and L. i. Vold.** 2010. Norovirus outbreaks linked to oyster consumption in the United Kingdom, Norway, France, Sweden and Denmark, 2010. Eurosurveillance **15**.
  229. **Whiting, R. C.** 1995. Microbial Modeling in Foods. Critical Reviews in Food Science and Nutrition **35**:467-494.
  230. **Yamane, K., J. Asato, N. Kawade, H. Takahashi, B. Kimura, and Y. Arakawa.** 2004. Two cases of fatal necrotizing fasciitis caused by *Photobacterium damsela* in Japan. Journal of Clinical Microbiology **42**:1370-1372.
  231. **Yang, Z.-Q., X.-A. Jiao, P. Li, Z.-M. Pan, J.-L. Huang, R.-X. Gu, W.-M. Fang, and G.-X. Chao.** 2009. Predictive model of *Vibrio parahaemolyticus* growth and

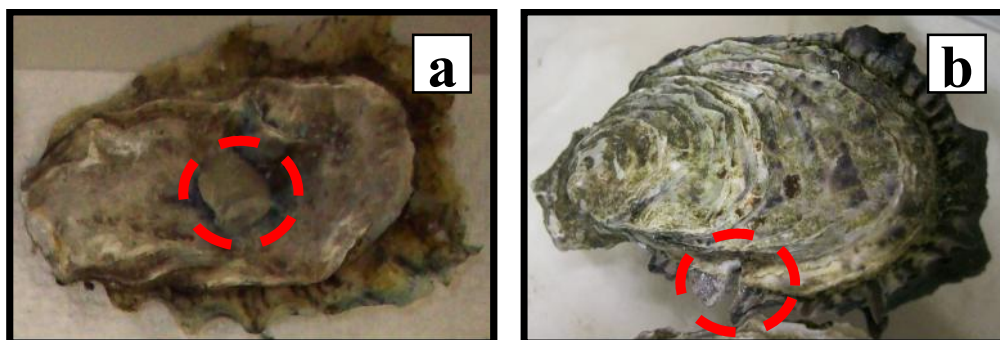
- survival on salmon meat as a function of temperature. *Food Microbiology* **26**:606-614.
232. **Yeung, P. S., and K. J. Boor.** 2004. Epidemiology, pathogenesis, and prevention of foodborne *Vibrio parahaemolyticus* infections. *Foodborne Pathogens and Disease* **1**:74-88.
  233. **Yoon, K. S., K. J. Min, Y. J. Jung, K. Y. Kwon, J. K. Lee, and S. W. Oh.** 2008. A model of the effect of temperature on the growth of pathogenic and nonpathogenic *Vibrio parahaemolyticus* isolated from oysters in Korea. *Food Microbiology* **25**:635-641.
  234. **Yu, L., F. Kow, and D. Grewal.** 2004. Oyster consumption study in Australia: results of a 2003 survey. Australian Maritime College.
  235. **Zhang, J., L. Liu, M. WeiSong, L. M. Moga, and X. Zhang.** 2009. Development of temperature-managed traceability system for frozen and chilled food during storage and transportation. *Journal of Food Agriculture & Environment* **7**:28-31.
  236. **Zhao, J.-S., D. Manno, and J. Hawari.** 2009. *Psychrilyobacter atlanticus* gen. nov., sp. nov., a marine member of the phylum *Fusobacteria* that produces H<sub>2</sub> and degrades nitramine explosives under low temperature conditions. *International Journal of Systematic and Evolutionary Microbiology* **59**:491-497.
  237. **Zhilina, T. N., R. Appel, C. Probian, E. Llobet-Brossa, J. Harder, F. Widdel, and G. A. Zavarzin.** 2004. *Alkaliflexus imshenetskii* gen. nov. sp. nov., a new alkaliphilic gliding carbohydrate-fermenting bacterium with propionate formation from a soda lake *Archives of Microbiology* **182**:244-253.
  238. **Zimmerman, A. M., A. DePaola, J. C. Bowers, J. A. Krantz, J. L. Nordstrom, C. N. Johnson, and D. J. Grimes.** 2007. Variability of total and pathogenic *Vibrio parahaemolyticus* densities in northern Gulf of Mexico water and oysters. *Applied and Environmental Microbiology* **73**:7589-7596.
  239. **Zwietering, M. H., and M. J. Nauta.** 2007. Predictive models in microbiological risk assessment, p. 110-125. *In* S. Brul, S. van Gerwen, and M. H. Zwietering (ed.), *Modelling microorganisms in food*. Woodhead Publishing Limited and CRC Press LLC, USA.

## Appendix A - *Vibrio parahaemolyticus* in *C. gigas* temperature model

### A1. Oyster injection test



**Figure A1.1.** *V. parahaemolyticus* growth profiles for artificially contaminated Pacific oysters stored at 20 and 25°C injected in the visceral mass (■) and adductor muscle (▣). Levels of natural *Vibrio* spp. in sea-water injected oysters in the visceral mass (▲) and adductor muscle (▲). The dashed line indicates the limit of detection by direct plating (2.8 log<sub>10</sub> CFU/g).



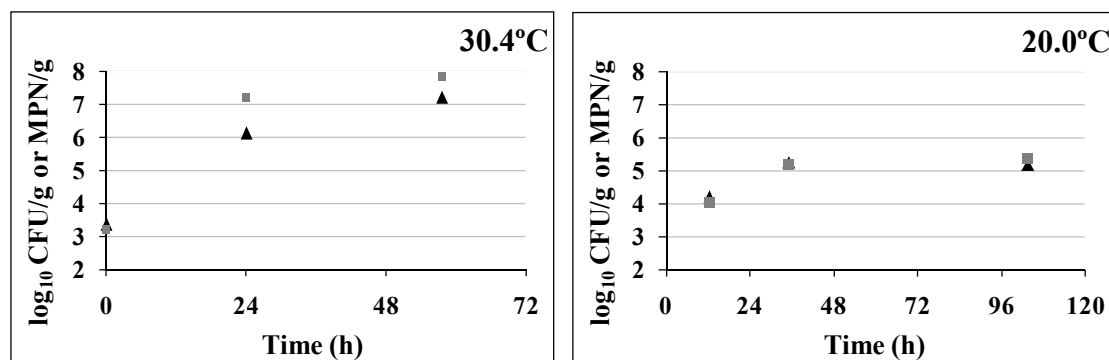
**Figure A1.2.** Pacific oysters showing the injected site for visceral mass (a) and adductor muscle (b).

## A2. TCBS and conventional MPN comparison for

### *Vibrio parahaemolyticus* enumeration

**Table A2.1.** *V. parahaemolyticus* enumeration for two different samples of three oysters using direct plating on TCBS or conventional MPN at different times after incubation at 20.0 and 30.4°C. Student's *t* test (significant level of 0.05,  $P > 0.05$ )

Storage conditions		Direct plating on TCBS		Conventional MPN		Student's <i>t</i> test (significance level 0.05) P value
T (°C)	Time (h)	CFU/g	log <sub>10</sub> (CFU/g)	MPN/g	log (MPN/g)	
30.4°C	0	2,520	3.40	2,400	3.38	0.47
	0	2,130	3.33	1,100	3.04	
	24	2,170,000	6.34	24,000,000	7.38	0.06
	24	850,000	5.93	11,000,000	7.04	
	57.5	18,800,000	7.27	110,000,000	8.04	0.08
	57.5	14,800,000	7.17	46,000,000	7.66	
20.0°C	12.25	13,500	4.13	11,000	4.04	0.16
	12.25	19,500	4.29	11,000	4.04	
	35.25	154,000	5.19	210,000	5.32	0.64
	35.25	226,000	5.35	110,000	5.04	
	103.5	91,000	4.96	46,000	4.66	0.83
	103.5	269,000	5.43	1,200,000	6.08	



**Figure A2.1.** *V. parahaemolyticus* growth profiles for Pacific oysters stored at 20.0 and 30.4°C enumerated by direct plating on TCBS (▲) or by conventional MPN method (■).

Mean data for each time interval shown in Table A2.1 are used in the graphs.

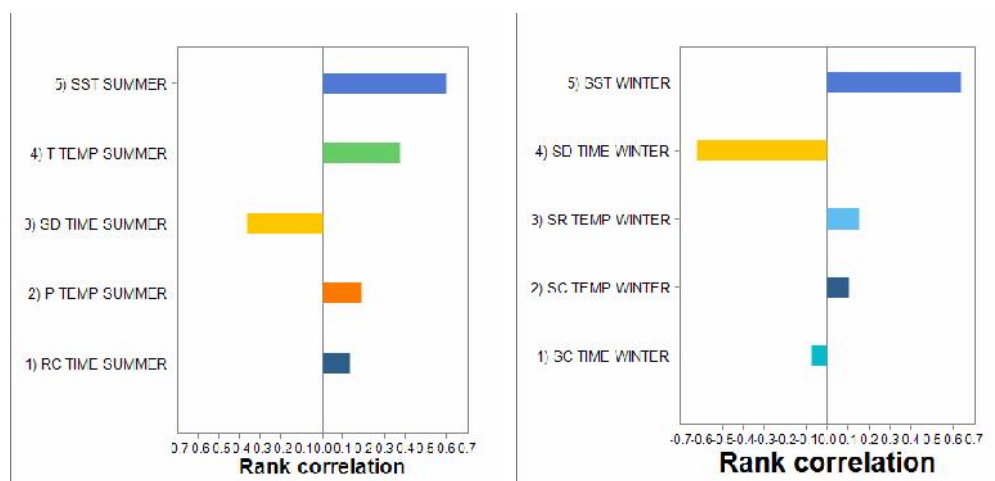
### A3. *V. parahaemolyticus* and TVC secondary model data analysis

**Table A3.1.** Regression data analyses of the secondary models.

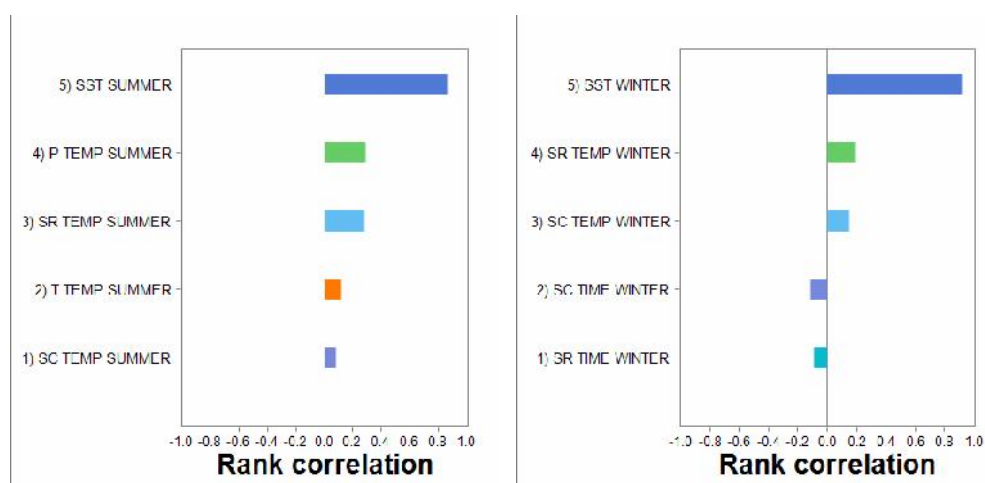
Eq.	Microorganism	Type	Regression	Coefficients	Standard Error	t Statistic	P-value	Lower 95%	Upper 95%
3	<i>V. parahaemolyticus</i>	Growth	Intercept	-0.405	0.117	-3.447	0.041	-0.779	-0.031
			x variable	0.030	0.005	5.815	0.010	0.014	0.047
4	<i>V. parahaemolyticus</i>	Inactivation	Intercept	-20.131	5.472	-3.679	0.067	-43.676	3.413
			x variable	4,131.229	1,538.137	2.686	0.115	-2,486.839	10,749.296
5	<i>Total viable bacteria count</i>	Growth	Intercept	0.069	0.020	3.395	0.015	0.019	0.118
			x variable	0.010	0.001	9.270	0.000	0.008	0.013

## Appendix B – Cold chain management tool for oysters: a case study

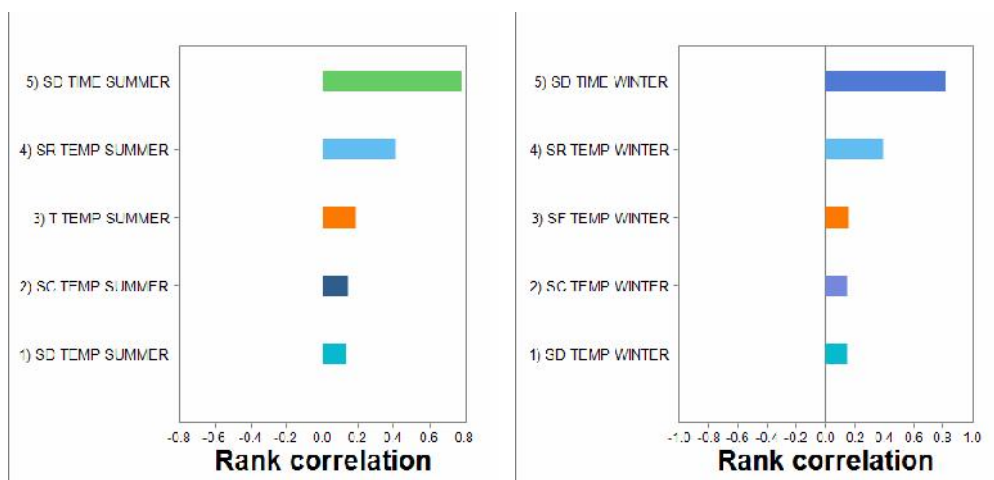
### B1. Sensitivity analyses for the simulation



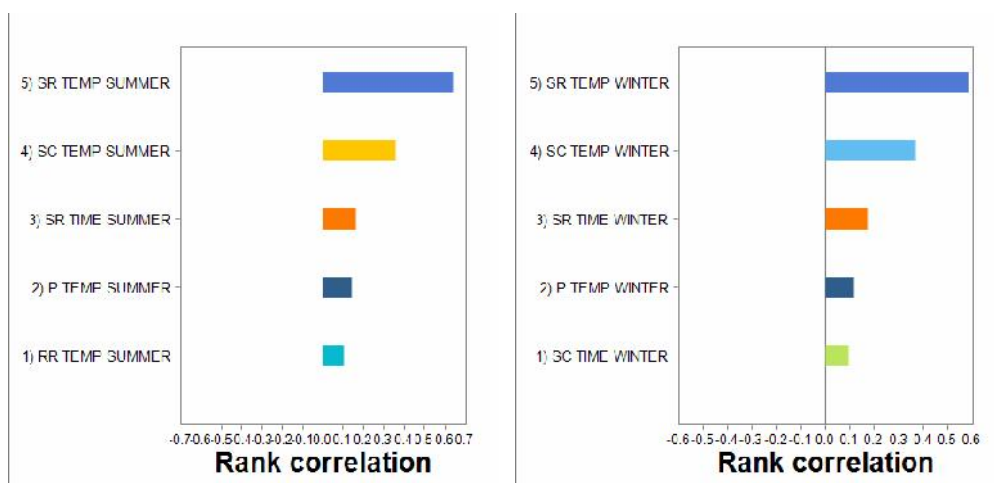
**Figure B1.1.** Tornado plot of model inputs for *V. parahaemolyticus* levels at consumer storage for the long supply chain in summer (left) and winter (right).



**Figure B1.2.** Tornado plot of model inputs for *V. parahaemolyticus* levels at consumer storage for the short supply chain in summer (left) and winter (right).



**Figure B1.3.** Tornado plot of model inputs for total viable bacteria count levels at consumer storage for the long supply chain in summer (left) and winter (right).



**Figure B1.4.** Tornado plot of model inputs for total viable bacteria count levels at consumer storage for the short supply chain in summer (left) and winter (right).



## Appendix C - Storage temperature effects on bacterial communities in *C. gigas*

### C1. Clone libraries analysis in fresh and stored Pacific oysters

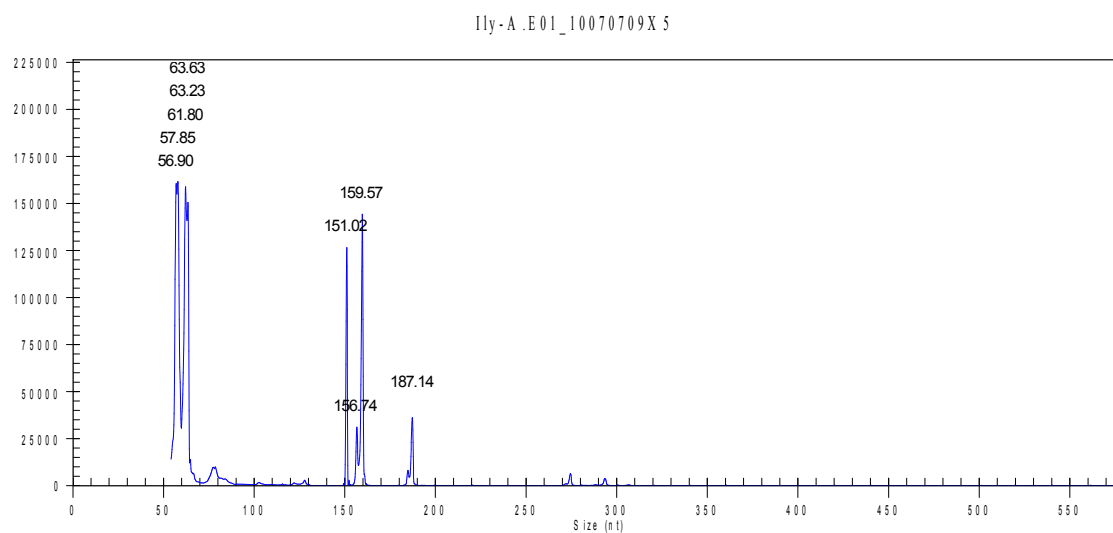
**Table C1.1.** Summary of closest related genus to the clones from the different oyster samples depending on library sample

Sample identification (closest related genus)			Clones depending on library sample											
			Number of clones						Percentage of clones (%)					
			F4	F15	F30	S4	S15	S30	F4	F15	F30	S4	S15	S30
1	<i>Spirochaetes</i>	<i>Spirochaeta</i>	7	3	6	1	2	3	9	5	7	1	2	3
2	<i>Spirochaetes</i>	<i>Cristispira</i>	4	0	3	4	1	0	5	0	3	4	1	0
3	<i>Alphaprot.</i>	<i>Sphingomonas</i>	6	0	3	3	6	0	8	0	3	3	7	0
4	<i>Alphaprot.</i>	<i>Thalassospira</i>	7	12	12	8	1	1	9	21	14	9	1	1
5	<i>Alphaprot.</i>	<i>Mesorhizobium</i>	0	0	18	0	2	0	0	0	21	0	2	0
6	<i>Alphaprot.</i>	<i>Prostheco- microbium</i>	0	0	2	0	0	0	0	0	2	0	0	0
7	<i>Alphaprot.</i>	<i>Roseobacter</i>	0	2	0	0	0	0	0	4	0	0	0	0
8	<i>Alphaprot.</i>	<i>Tistrella</i>	0	0	1	0	0	0	0	0	1	0	0	0
9	<i>Alphaprot.</i>	<i>Octadecabacter</i>	0	0	1	0	0	0	0	0	1	0	0	0
10	<i>Alphaprot.</i>	<i>Candidatus Odysella</i>	0	0	1	0	0	0	0	0	1	0	0	0
11	<i>Alphaprot.</i>	<i>Anaplasma</i>	3	0	0	1	0	0	4	0	0	1	0	0
12	<i>Alphaprot.</i>	<i>Candidatus Midichloria</i>	1	0	0	0	0	0	1	0	0	0	0	0
13	<i>Alphaprot.</i>	<i>Rickettsia</i>	1	0	0	1	0	0	1	0	0	1	0	0
14	<i>Alphaprot.</i>	<i>Rhodobacter</i>	1	0	0	0	0	0	1	0	0	0	0	0
15	<i>Alphaprot.</i>	<i>Filomicrobium</i>	1	0	0	0	0	0	1	0	0	0	0	0
16	<i>Alphaprot.</i>	<i>Loktanella</i>	1	0	0	0	1	0	1	0	0	0	1	0
17	<i>Alphaprot.</i>	<i>Azospirillum</i>	1	0	0	0	0	0	1	0	0	0	0	0
18	<i>Alphaprot.</i>	<i>Amaricoccus</i>	1	0	0	1	0	0	1	0	0	1	0	0

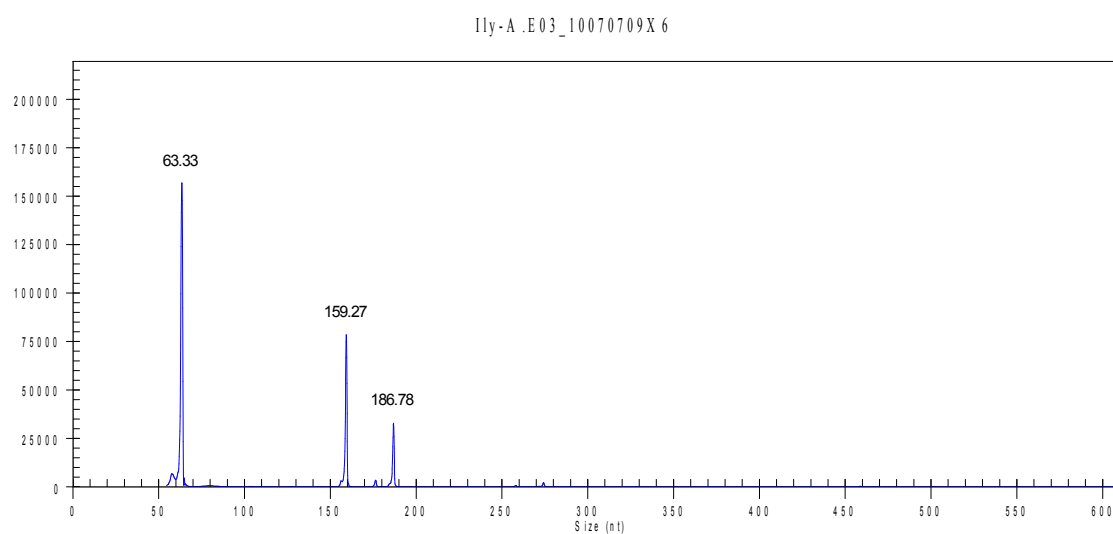
Sample identification (closest related genus)			Clones depending on library sample											
			Number of clones						Percentage of clones (%)					
			F4	F15	F30	S4	S15	S30	F4	F15	F30	S4	S15	S30
19	<i>Alphaprot.</i>	<i>Porphyrobacter</i>	0	0	0	1	0	0	0	0	0	1	0	0
20	<i>Alphaprot.</i>	<i>Ehrlichia</i>	1	0	0	0	0	0	1	0	0	0	0	0
21	<i>Alphaprot.</i>	<i>Paracoccus</i>	0	1	0	0	0	0	0	2	0	0	0	0
22	<i>Alphaprot.</i>	<i>Sulfitobacter</i>	0	1	1	0	0	1	0	2	1	0	0	1
23	<i>Alphaprot.</i>	<i>Sphingopyxis</i>	0	1	0	0	0	0	0	2	0	0	0	0
24	<i>Betaprot.</i>	<i>Polynucleobacter</i>	4	2	2	6	0	0	5	4	2	7	0	0
25	<i>Betaprot.</i>	<i>Alcaligenes</i>	0	0	1	1	0	0	0	0	1	1	0	0
26	<i>Betaprot.</i>	<i>Burkholderia</i>	0	1	0	0	0	0	0	2	0	0	0	0
27	<i>Epsilonprot.</i>	<i>Arcobacter</i>	0	0	0	2	0	5	0	0	0	2	0	6
28	<i>Epsilonprot.</i>	<i>Helicobacter</i>	0	1	0	0	0	0	0	2	0	0	0	0
29	<i>Deltaprot.</i>	<i>Geopsychrobacter</i>	1	0	0	0	0	0	1	0	0	0	0	0
30	<i>Deltaprot.</i>	<i>Desulfuromonas</i>	1	0	0	0	0	0	1	0	0	0	0	0
31	<i>Gammaprot.</i>	<i>Legionella</i>	1	0	0	0	0	0	1	0	0	0	0	0
32	<i>Gammaprot.</i>	<i>Thiopfundum</i>	1	0	0	0	0	0	1	0	0	0	0	0
33	<i>Planctomycetes</i>	<i>Rhodopirellula</i>	0	3	0	0	0	0	0	5	0	0	0	0
34	<i>Planctomycetes</i>	<i>Pirellula</i>	0	0	1	0	0	0	0	0	1	0	0	0
35	<i>Verrucomicrobia</i>	<i>Pelagicoccus</i>	0	0	1	0	0	0	0	0	1	0	0	0
36	<i>Verrucomicrobia</i>	<i>Coralimargarita</i>	1	0	0	0	0	0	1	0	0	0	0	0
37	<i>Fusobacteria</i>	<i>Ilyobacter, Psychrilyobacter</i>	9	2	2	39	11	18	11	4	2	44	12	20
38	<i>Fusobacteria</i>	<i>Fusobacterium</i>	0	0	0	0	0	1	0	0	0	0	0	1
39	<i>Fusobacteria</i>	<i>Cetobacterium</i>	0	0	0	0	1	0	0	0	0	0	1	0
40	<i>Firmicutes</i>	<i>Bacillus</i>	0	0	0	1	0	0	0	0	0	1	0	0
41	<i>Firmicutes</i>	<i>Eubacterium</i>	0	2	0	0	0	0	0	4	0	0	0	0
42	<i>Tenerictues</i>	<i>Mycoplasma</i>	1	3	1	3	2	0	1	5	1	3	2	0
43	<i>Tenerictues</i>	<i>Spiroplasma</i>	3	3	3	1	4	1	4	5	3	1	4	1
44	<i>Tenerictues</i>	<i>Haloplasma</i>	0	0	1	0	0	0	0	0	1	0	0	0
45	<i>Cyanobacteria</i>	<i>Synechococcus</i>	6	10	9	2	0	4	8	18	10	2	0	5
46	<i>Bacteroidetes</i>	<i>Alkaliflexus</i>	0	0	0	0	31	7	0	0	0	0	34	8
47	<i>Bacteroidetes</i>	<i>Dokdonia</i>	0	0	0	1	0	5	0	0	0	1	0	6

Sample identification (closest related genus)			Clones depending on library sample											
			Number of clones						Percentage of clones (%)					
			F4	F15	F30	S4	S15	S30	F4	F15	F30	S4	S15	S30
48	<i>Bacteroidetes</i>	<i>Psychroserpens</i>	0	0	0	0	1	5	0	0	0	0	1	6
49	<i>Bacteroidetes</i>	<i>Bizionia</i>	0	0	0	2	4	4	0	0	0	2	4	5
50	<i>Bacteroidetes</i>	<i>Polaribacter</i>	0	2	0	3	2	7	0	4	0	3	2	8
	<i>Bacteroidetes</i>	<i>Cytophaga</i> <i>transferred to</i>	0	0	0	0	4	4	0	0	0	0	4	5
51	<i>Bacteroidetes</i>	<i>Eudoraea</i>	0	0	1	0	0	0	0	0	1	0	0	0
52	<i>Bacteroidetes</i>	<i>Tenacibaculum</i>	1	0	0	0	1	1	1	0	0	0	1	1
53	<i>Bacteroidetes</i>	<i>Salegentibacter</i>	0	0	0	0	1	1	0	0	0	0	1	1
54	<i>Bacteroidetes</i>	<i>Cellulophaga</i>	0	0	0	1	0	0	0	0	0	1	0	0
55	<i>Bacteroidetes</i>	<i>Actibacter</i>	1	0	0	0	0	0	1	0	0	0	0	0
56	<i>Bacteroidetes</i>	<i>Algibacter</i>	1	0	0	0	0	0	1	0	0	0	0	0
57	<i>Bacteroidetes</i>	<i>Winogradskyella</i>	1	0	0	0	0	0	1	0	0	0	0	0
58	<i>Bacteroidetes</i>	<i>Fulvivirga</i>	1	0	0	0	0	0	1	0	0	0	0	0
59	<i>Bacteroidetes</i>	<i>Gilvibacter</i>	1	0	1	0	0	0	1	0	1	0	0	0
60	<i>Bacteroidetes</i>	<i>Flavobacterium</i>	2	0	1	1	3	3	3	0	1	1	3	3
61	<i>Bacteroidetes</i>	<i>Ulvibacter</i>	0	0	0	2	0	0	0	0	0	2	0	0
62	<i>Bacteroidetes</i>	<i>Gramella</i>	0	0	0	0	2	2	0	0	0	0	2	2
63	<i>Bacteroidetes</i>	<i>Prolixibacter</i>	0	0	0	0	3	0	0	0	0	0	3	0
64	<i>Bacteroidetes</i>	<i>Robiginitalea</i>	0	0	1	0	0	0	0	0	1	0	0	0
65	<i>Bacteroidetes</i>	<i>Croceitalea</i>	1	0	0	0	0	0	1	0	0	0	0	0
66	<i>Bacteroidetes</i>	<i>Fluviicola</i>	0	0	1	0	0	3	0	0	1	0	0	3
67	<i>Bacteroidetes</i>	<i>Krokinobacter</i>	0	0	0	0	0	1	0	0	0	0	0	1
68	<i>Bacteroidetes</i>	<i>Lishizhenia</i>	0	0	0	0	0	1	0	0	0	0	0	1
69	<i>Bacteroidetes</i>	<i>Lacinutrix</i>	0	0	0	0	0	1	0	0	0	0	0	1
70	<i>Bacteroidetes</i>	<i>Lutimonas</i>	0	0	0	0	0	1	0	0	0	0	0	1
71	<i>Bacteroidetes</i>	<i>Bacteroides</i>	0	0	0	0	0	1	0	0	0	0	0	1
72	<i>Bacteroidetes</i>	<i>Persicivirga</i>	0	0	0	0	0	2	0	0	0	0	0	2
73	<i>Bacteroidetes</i>	<i>Olleya</i>	0	0	0	0	0	1	0	0	0	0	0	1

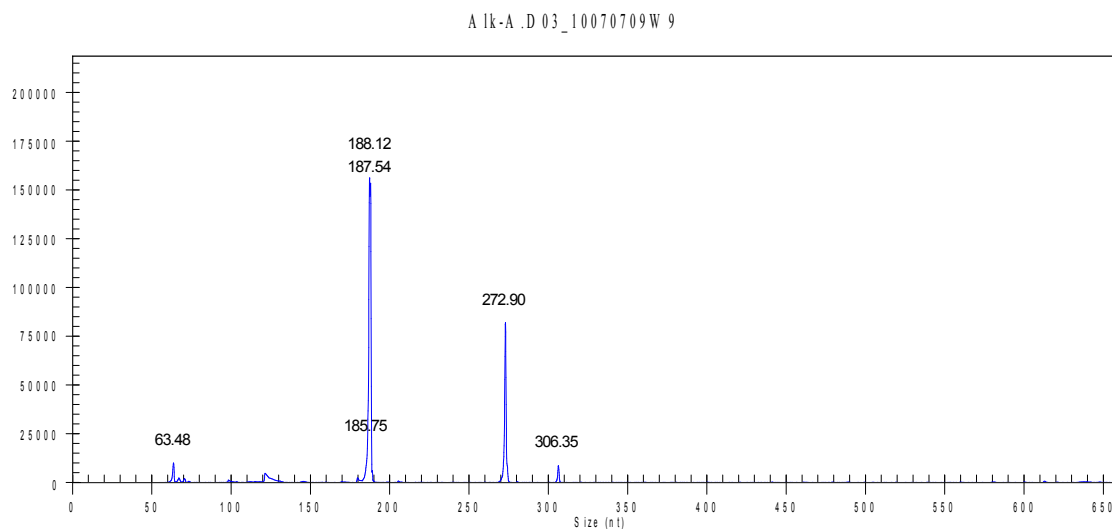
Fresh (F) and stored (S) Pacific oysters at 4, 15 and 30°C

**C2. TRFLP profiles for clones**

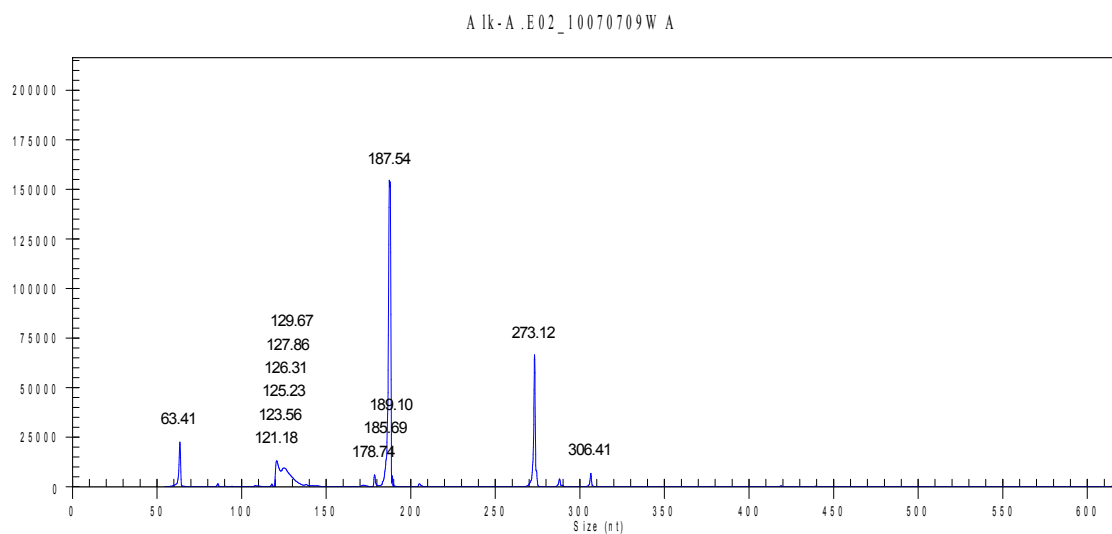
**Figure C2.1.** TRFLP profile for clone (JF827404) identified as *Psychrilyobacter* spp.



**Figure C2.2.** TRFLP profile for clone (JF827561) identified as *Psychrilyobacter* spp.



**Figure C2.3.** TRFLP profile for clone (JF827488) identified as *Alkaliflexus*-related spp.



**Figure C2.4.** TRFLP profile for clone (JF827564) identified as *Alkaliflexus*-related spp.